An integrated “omics” approach to unravel the impact of root symbionts on tomato direct and indirect defenses against insect herbivores

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Contents

Introduction

Plant defense mechanisms

Plants fix the solar energy that drives nearly all living processes on Earth; therefore, they are central players in complex food webs (Pieterse et al., 2014). However, as sessile organisms they cannot escape stressful conditions generated in their physical environment (abiotic stress) or due to their interaction with herbivorous insects and pathogenic microorganisms, such as fungi and bacteria (biotic stress) (Kissoudis et al., 2014). The evolutionary arms race between plants and their attackers has resulted in the development of a sophisticated defense system in plants that has the ability to recognize non-self molecules as well as signals from damaged cells, and subsequently activates plant immune responses against them (Hare, 2011; Howe & Jander, 2008; Nishad et al., 2020; Verhage et al., 2010).

Plants confront herbivores both directly and indirectly. Direct plant defenses are mediated by plant physical structures that affect the herbivore's physiology, such as mechanical protection on the plant surface (i.e., thorns, trichomes, waxy cuticle, cell wall) (Figure 1), or the synthesis of toxic chemicals (i.e. alkaloids, terpenoids, anthocyanins, phenols) and proteins that either kill or impede the development of herbivores (Hanley et al., 2007). Indirect plant defenses act through the attraction of natural enemies of the attacking herbivores (Heil, 2008). The release of volatile organic compounds (VOCs), consisting mainly of fatty acid derivatives, terpenoids, and aromatic compounds by herbivore-infested plants, can attract parasitoids and predators of the herbivores (Figure 2) (De Moraes et al., 1998; Dicke & Sabelis, 1988; Dicke et al., 1990; Kessler & Baldwin, 2001). The mode of damage on the plant determines the composition of the volatile blends emitted (Hilker & Meiners, 2006; Mithöfer et al., 2005). Moreover, VOCs are emitted not only aboveground, but also belowground in the rhizosphere. Therefore, VOCs can be considered as info-chemicals that mediate a plethora of interactions of plants with other species both above- and belowground (Bezemer & van Dam, 2005; Dam et al., 2016). Indirect defenses can also be conferred by plant traits that accommodate natural enemies, such as domatia, or extrafloral nectar that provide the natural enemies of herbivores with shelter and food, respectively (Heil, 2015; Wäckers et al., 2005).

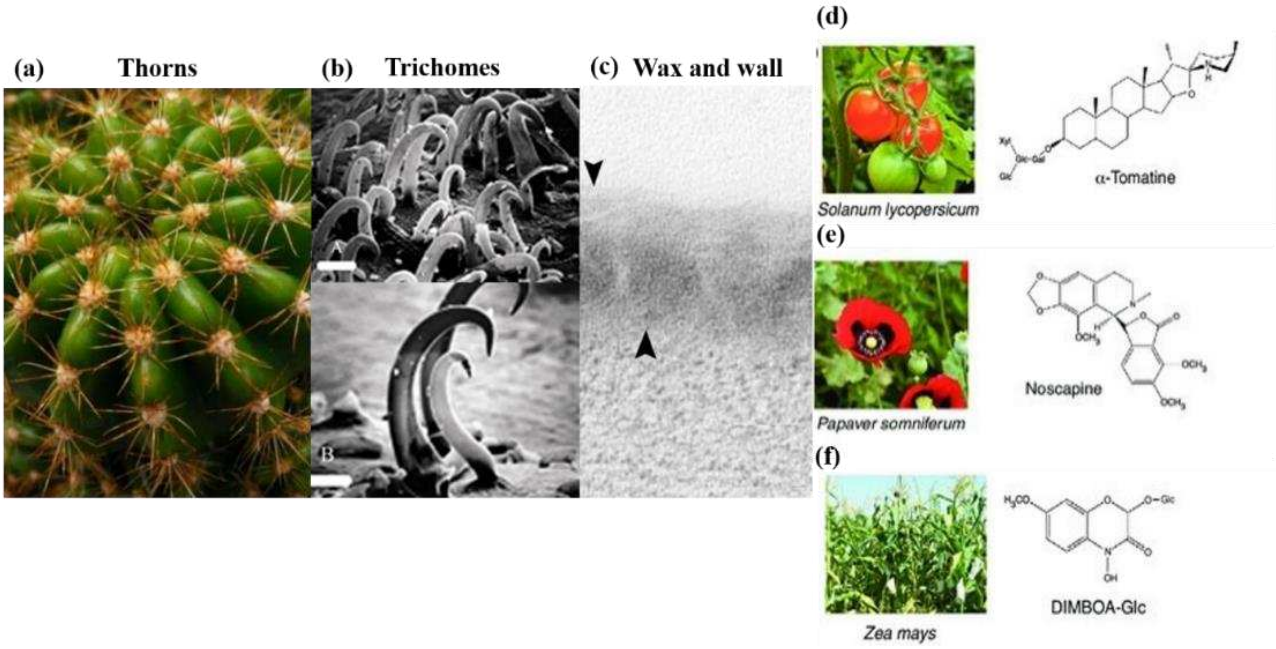

Figure 1. Examples of plant physical structures and specialized plant-synthesized metabolites that mediate plant defenses against herbivores. (a) thorns, (b) trichomes, (c) waxy cuticle and cell wall, (d) the steroidal glycoalkaloid *α*-tomatine (tomato; *Solanum lycopersicum*), (e) the alkaloid noscapine (poppy; *Papaver somniferum*), (f) the cyclic hydroxamic acid 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) (maize; *Zea mays*). Figures (a), (b) and (c) found in Link ("Plants and Arthropods: Friends or Foes?," 2011). Figures (d), (e) and (f) adapted from Nützmann and Osbourn (2014).

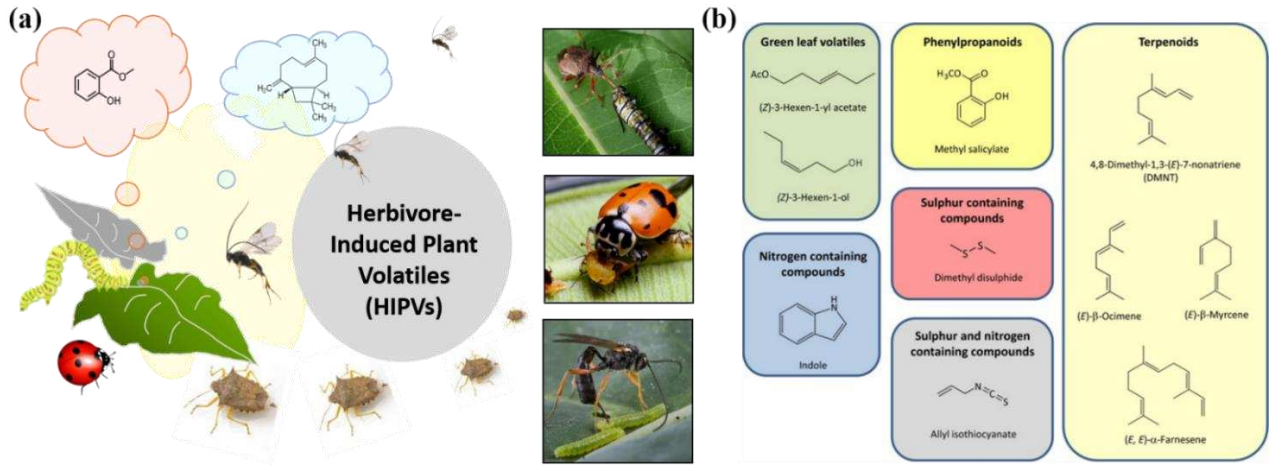

Figure 2. The emission of herbivore-induced plant volatiles (HIPVs) represents a form of indirect plant defense responses against insect herbivores. (a) The release of HIPVs mediates the attraction of the herbivore's natural enemies (parasitoids and predators), (b) main chemical classes of HIPVs emitted in response to plant herbivory. Figure (a) found in Link (Pozo et al., 2020). Source of figure (b): (Aartsma et al., 2017).

Perception of insect herbivory by plants

Upon insect attack, the host plant perceives at least two types of signals: (1) physical injury or wounding, known as damage-associated molecular patterns (DAMPs) and (2) chemical cues found in herbivore oral secretions (OS) or oviposition fluids (OF), known as herbivore-associated molecular patterns (HAMPs) (Erb & Reymond, 2019; Felton et al., 2014; Wu & Baldwin, 2010). Herbivore-plant interactions are generally initiated at the plant cell membrane, where herbivore-associated elicitors (HAEs) trigger a series of signaling cascades, which initiate induced plant responses (Arimura et al., 2009; Arimura et al., 2011; Maffei et al., 2007a, 2007b). As it has been proposed, the foremost event following insect attack is the plasma membrane potential change (V_m) (Bricchi et al., 2010; Zebelo & Maffei, 2012), followed by the generation of secondary messengers such as cytosolic calcium (Ca^{2+}) and reactive oxygen species (ROS) (Halliwell & Gutteridge, 2015; Shin et al., 2005; Steffens et al., 2013) that facilitate plant defense signal transduction. This leads to a suit of defense-related traits, including the induction of trichomes, spines and secondary metabolites (i.e., alkaloids, phenolics, VOCs) that negatively affect herbivore fitness and mediate multi-trophic interactions (Kariyat et al., 2012; Kaur & Kariyat, 2020; Turlings & Erb, 2018) (Figure 3). Hormonal signaling networks are major mediators between herbivory perception and early defense signaling, resulting in the induction of plant defense genes. Induced direct and indirect defenses are regulated primarily by the action of the phytohormones jasmonic acid (JA), ethylene (ET) and/or salicylic acid (SA) (Diezel et al., 2009; Erb et al., 2012). The hormone signaling hubs intersect and can interact in an antagonistic or

synergistic manner allowing plants to fine-tune their defenses depending on the threat (Erb et al., 2012; Takatsuji & Jiang, 2014).

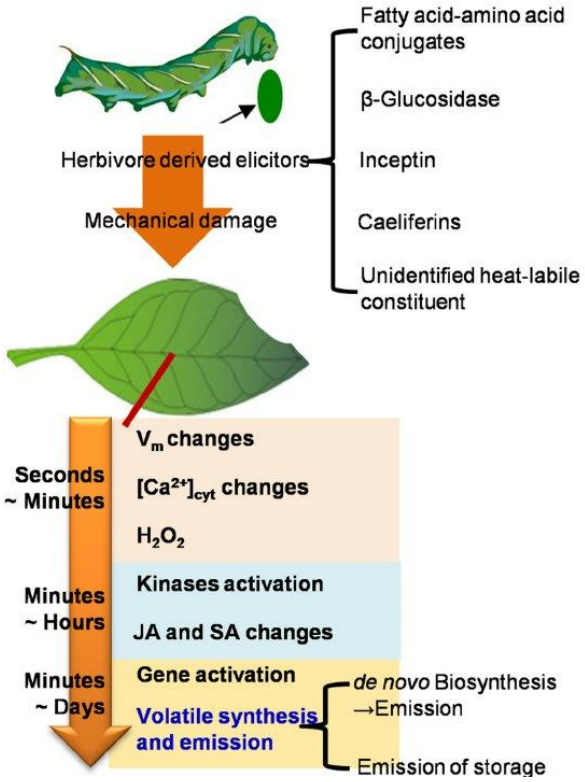

Figure 3. Perception of insect herbivory by plants. The cascade of events taking place from the onset of herbivore attack until volatile emission (Dong et al., 2016). Figure based on (Maffei et al., 2007a).

There is evidence that the herbivore's feeding guild can influence the induction of specific phytohormonal pathways (Heidel & Baldwin, 2004). For instance, plant responses to puncture-feeders appear to be primarily regulated by the SA pathway; those to phloem-feeders by both SA and JA pathways; and responses to leaf-chewing lepidopterans have been shown to be predominantly regulated by the JA pathway (Mithöfer et al., 2009). However, other studies point at a simultaneous activation of two or all of these signaling pathways by lepidopteran herbivores (Van Poecke & Dicke, 2004). Moreover, changes in the induction of signaling patterns (both SA and JA pathways) have been detected because of plants being attacked simultaneously by herbivores of one or more feeding guilds, or because of insect herbivore-pathogen interactions (Thaler et al., 2010). Therefore, despite the fact that transcriptional responses can be tailored depending on the plant-insect herbivore interaction (in case that plants are attacked by insects belonging to the same feeding guild), there can be substantial overlap of defense-related gene expression profiles, when plants experience simultaneous attack by herbivores belonging to different feeding guilds (Howe & Jander, 2008).

Impact of beneficial root microbes on plant defense mechanisms

Apart from microbial pathogens and insect herbivores, plants also nurture a vast community of commensal and mutualistic microbes that provide them with essential services, such as improved mineral uptake, nitrogen fixation, growth promotion, and protection from pathogens (Lugtenberg & Kamilova, 2009; Shoresh et al., 2010). This plant microbiota is predominantly hosted in the rhizosphere, where up to 40% of the plant's photosynthetically fixed carbon is deposited. Several genera of the rhizosphere microbiota, which are referred to as plant growth-promoting rhizobacteria (PGPR) and fungi (PGPF), are able to enhance plant growth and improve plant health (Lugtenberg & Kamilova, 2009; Shoresh et al., 2010).

Microbe-induced systemic resistance (ISR) in plants

Induced resistance (IR) is a physiological state of enhanced plant defensive capacity, which is triggered by biological or chemical inducers, and protects the plant tissues (that are not exposed to the initial attack), from a future attack by pathogens and herbivorous insects (Van Loon et al., 1998). IR can be triggered in plants due to infection by pathogens, in response to insect herbivory, or upon root colonization by PGPR and PGPF. Colonization of plant roots by mutualistic microbes (such as PGPR and PGPF) results in a physiological state called induced systemic resistance (ISR) (Romera et al., 2019). The discovery of ISR occurred around 1991, when several studies reported that root colonization by certain non-pathogenic bacteria promoted plant health by the stimulation of plant defense responses [reviewed by (Pieterse et al., 2014)]. After these pioneering works with PGPR, ISR was further extended to PGPF, such as *Trichoderma* spp.*, Piriformospora indica,* non-pathogenic *Fusarium* strains, and to Arbuscular Mycorrhiza Fungi (AMF) as well (Alizadeh et al., 2013; Biles & Martyn, 1989; Cordier et al., 1998; Fuchs et al., 1997; Khaosaad et al., 2007; Nouayti et al., 2018; Pieterse et al., 2014; Pozo et al., 2002; Segarra et al., 2009; Zhu & Yao, 2004).

Several microbial elicitors have been proposed to be responsible for the onset of ISR. Among these elicitors, microbe-associated molecular patterns (MAMPs), VOCs, and siderophores are found (del Carmen Orozco-Mosqueda et al., 2013; Garnica-Vergara et al., 2016; Martínez-Medina et al., 2017; Villena et al., 2018; Zamioudis et al., 2015). MAMPs, like flagellin, chitin and lipopolysaccharides (LPS), are conserved microbial molecules released by beneficial microbes (Pieterse et al., 2014; Villena et al., 2018; Zeidler et al., 2004). The VOCs derived from beneficial microbes are capable of triggering drastic changes in plant growth patterns, generally by altering hormone signaling (Garnica-Vergara et al., 2016; Martínez-Medina et al., 2017; Sharifi & Ryu, 2018; Tyagi et al., 2018). Siderophores are iron (Fe) chelating agents released by bacteria to further acquire Fe from the medium (Aznar et al., 2014; Aznar et al., 2015; Aznar & Dellagi, 2015). MAMPs are perceived by pattern recognition receptors (PRRs), whereas the other elicitors might be perceived by other receptors, which are not known in all cases (Aznar et al., 2015; Aznar & Dellagi, 2015; Jankiewicz & Kołtonowicz, 2012; Sharifi & Ryu, 2018). Upon perception, the elicitors trigger ISR by modulating diverse plant hormones, which act as central players in the

plant immune signaling network, resulting in the activation of defense responses (Pieterse et al., 2012; Pieterse et al., 2014; Sharifi & Ryu, 2018; Tyagi et al., 2018). Among the hormones involved in ISR, JA, SA, abscisic acid (ABA), ET, auxin and nitric oxide (NO) play a major role (Acharya et al., 2011; Camehl et al., 2010; Garnica-Vergara et al., 2016; Md Motaher Hossain et al., 2017; Knoester et al., 1999; Martínez-Medina et al., 2013; Nascimento et al., 2018; Nie et al., 2017; Pieterse et al., 2014; Shoresh et al., 2005; Ioannis A. Stringlis et al., 2018; Ton et al., 2001; Zhang et al., 2007).

Priming of microbe-induced plant defenses

In most cases, ISR is associated with a potentiated defensive capacity, which is termed "defense priming" (Nie et al., 2017). Defense priming enables plant cells to respond to very low levels of a stimulus in a more rapid and robust manner compared to non-primed cells (Conrath, 2009; Conrath et al., 2006; Conrath et al., 2002). Priming can be elicited by pathogens, herbivores, beneficial microbes, and selected synthetic compounds (i.e., benzothiadiazole [BTH] and β-aminobutyric acid [BABA]) (Conrath et al., 2006; Frost et al., 2008; Heil & Silva Bueno, 2007). Plants that are primed for enhanced defense do not express defenses in the absence of an attacker but show a faster and stronger activation of cellular defense responses upon attack compared to non-primed control plants (Conrath et al., 2006; Conrath et al., 2002; Frost et al., 2008). Prior activation of defenses is not a prerequisite for the primed state, which makes priming a cost-effective form of induced immunity. Another benefit of priming is that it provides plant resistance against a broad spectrum of attackers.

Several mechanisms underlying priming have been reported (Vos et al., 2013). Inactive cellular proteins that play a role in cellular signal amplification have been shown to accumulate in primed plants, where they remain dormant until activation by stressors, resulting in an accelerated response. For instance, such dormant signal transducers involved in priming are transcription factors (TFs) and mitogen-activated protein kinases [MAPKs; (Beckers et al., 2009; Pozo et al., 2008; Van der Ent et al., 2009)]. In addition, chromatin modifications at the promoters of priming-associated genes have also been shown to be involved in the regulation of the primed state (Jaskiewicz et al., 2011; Luna et al., 2012; Rasmann et al., 2012). In several cases, priming has been demonstrated to be transferred to the plant's offspring, which is in some cases linked to epigenetic changes, allowing plants to retain memory of a threatening situation into one or more successive plant generations (Luna et al., 2012; Pieterse et al., 2012; Rasmann et al., 2012; Slaughter et al., 2012).

Regulation of microbe-mediated ISR

Several studies using *Arabidopsis* mutants impaired in JA or ET signaling have demonstrated that JA and ET are central players in the regulation of rhizobacteria-mediated ISR (Pieterse et al., 1998). For instance, the JA signaling mutants *jar1*, *jin1*, *coi1*, and diverse ET signaling mutants,

including *etr1*, *ein2*, *ein3*, and *eir1* were shown to be defective in *Pseudomonas fluorescenes* WCS417r-ISR (Knoester et al., 1999; Pieterse et al., 1998; Pozo et al., 2008). Furthermore, for many other PGPR, such as *Serratia marcescens* 90-166, *P. protegens* CHA0, *P. fluorescens* Q2-87; and PGPF such as *Penicillium sp.* GP16-2, *Trichoderma harzianum* T39, and *Piriformspora indica*, genetic evidence in *Arabidopsis* pointed to a role for JA and/or ET in the regulation of ISR (Ahn et al., 2007; Hossain et al., 2008; Iavicoli et al., 2003; Korolev et al., 2008; Ryu et al., 2004; Stein et al., 2008; Weller et al., 2012). However, in some particular cases, it has been reported that ISR requires SA accumulation (Alizadeh et al., 2013; Contreras-Cornejo et al., 2011; Martínez-Medina et al., 2013; Mathys et al., 2012; Ryu et al., 2003) as well. For instance, the non-expressor of pathogenesis-related gene 1 (NPR1) protein has also been shown as a key regulator for JA-/ET-regulated ISR triggered by *P. fluorescens* WCS417r (Pieterse et al., 1998), and many other PGPR and PGPF as well (Nie et al., 2017; Pieterse et al., 2014).

Besides plant hormones, an array of transcription factors (TFs) is also involved in ISR by beneficial microbes. Experiments using *Arabidopsis thaliana* plants revealed that the induction of systemic resistance was associated with enhanced transcription levels of a set of transcription factor genes, among which the APETALA2/ ETHYLENE RESPONSIVE FACTOR (AP2/ERF) family was significantly overrepresented (Van der Ent et al., 2009). Analysis of the promoter sequences of all the JA-responsive *Arabidopsis* genes, showing an induced expression pattern in ISR-expressing plants, revealed that the promoters of the ISR-enhanced genes were significantly enriched for a cis-acting G-box-like motif (Pozo et al., 2008). Notably, this motif is the binding site for the TF MYC2 that acts as a key transcriptional regulator of JA-dependent defenses (Memelink, 2009). Studies employing MYC2-impaired *Arabidopsis jin1* mutants highlighted the importance of this TF in regulating priming during ISR (Pozo et al., 2008; Stein et al., 2008). WRKY transcription factors are also involved in the regulation of plant-beneficial microbe interactions. For instance, WRKY11 and WRKY70 were shown to be involved in the regulation of *Bacillus cereus* strain AR156-triggered ISR in *Arabidopsis*, through the JA and SA signaling pathways, respectively (Jiang et al., 2016). The R2R3-type MYB transcription factor gene, MYB72, was identified as one of the most significantly induced genes in *Arabidopsis* roots in response to *P. fluorescens* WCS417r-triggered ISR (Verhagen et al., 2004). In particular, MYB72 was expressed at low levels in the root vascular bundle of non-induced plants. However, it was highly expressed in the root epidermis and cortical cells upon colonization by the ISR-inducing PGPR. Experiments using *Arabidopsis myb72* knockout mutants showed that these plants were unable to express ISR against foliar pathogens after treatment with *P. fluorescens* WCS417r or *P. putida* WCS358r, thus indicating that this root-specific TF is necessary for the onset of ISR. Furthermore, MYB72 was also shown induced in the roots of *Arabidopsis* colonized by *Trichoderma* spp., and it was thus considered as a crucial component of *Trichoderma* spp.-triggered ISR (Alizadeh et al., 2013; Brotman et al., 2013; Segarra et al., 2009). These two findings indicate that MYB72 is a node of convergence in the ISR signaling pathway triggered by different beneficial microbes (Pieterse et al., 2014).

Apart from its key role in the onset of ISR, the root-specific TF MYB72 is also essential for plant growth under Fe deficiency conditions (Segarra et al., 2009; Ioannis A Stringlis et al., 2018; Van der Ent et al., 2008; Verbon et al., 2017). The study of Zamioudis et al. (2015) showed that two Fe deficiency marker genes were co-regulated with *MYB72* in *Arabidopsis* roots colonized by *Pseudomonas* ISR-inducing strains, but not in the roots of plants colonized by a non-ISR-inducing

strain. This finding indicates that MYB72 is probably a link between rhizobacteria-mediated ISR and Fe deficiency responses (Pieterse et al., 2014; Verbon et al., 2017; Zamioudis et al., 2014). The signaling molecule NO is a highly reactive free radical implicated in plant responses to several biotic and abiotic stresses, including adaptation to low Fe availability (Chen et al., 2010; García et al., 2010; Graziano & Lamattina, 2007; Meiser et al., 2011), and defense responses against pathogen attack (Martínez-Medina et al., 2019; Molina-Moya et al., 2019). In addition, NO has been shown to accumulate in the roots of *Arabidopsis* and tomato plants inoculated with *Trichoderma* fungi, thus indicating that NO might hold an important role in the establishment of the plant-*Trichoderma* symbiosis (Gupta et al., 2014; Martínez-Medina et al., 2019). The study of Zamioudis et al. (2015) reported that NO signaling was involved in the initiation of MYB72-dependent Fe deficiency response, which was triggered by *Pseudomonas* spp., as part of the onset of rhizobacteria-mediated ISR. In regard to *Trichoderma* spp., Martínez-Medina et al. (2017) showed that perception of *Trichoderma*-produced VOCs enhanced the expression levels of *MYB72* in *Arabidopsis* and tomato plants, and enhanced the accumulation of NO in *Arabidopsis* roots. Interestingly, later, it was shown that the regulation of *MYB72* expression by *Trichoderma*-produced VOCs was dependent on the levels of NO in the roots of *Arabidopsis* (Pescador et al., 2022). Therefore, it was concluded that NO signaling in the roots is essential for the onset of ISR triggered by *Trichoderma*-emitted VOCs in *Arabidopsis* (Pescador et al., 2022).

Among the diverse rhizosphere microbial population, particular attention has been paid to several beneficial fungi due to their potential to enhance plant development and growth and protect plants against abiotic and biotic stresses. Over the last decades, a plethora of PGPF has been studied, including those belonging to the genera *Alternaria*, *Aspergillus*, *Chaetonium*, *Fusarium*, *Penicillium*, *Phoma*, *Serendipita* and *Trichoderma* (Md. Motaher Hossain et al., 2017; Hyde et al., 2019). Similarly, a large number of studies has investigated how plants benefit from their association with AMF in regard to development, nutrition, and stress alleviation [reviewed by (Diagne et al., 2020; Thirkell et al., 2017)]. Among beneficial fungi in the rhizosphere, the AMF and the PGPF *Trichoderma* strains hold a major role within the beneficial root symbionts studied and will be described in detail in the following sections of this study.

Arbuscular mycorrhizal fungi

Arbuscular mycorrhizal fungi (AMF) are soil microorganisms that form a symbiotic relationship with 80-90% of vascular plant species, and 90% of agricultural plants (Smith & Read, 2008) making mycorrhizal symbiosis the most widely spread beneficial interaction between plants and microorganisms (Parniske, 2008). AMF belong to the sub-kingdom of *Mucoromyceta* and the phylum *Glomeromycota* (Tedersoo et al., 2018). They are obligate biotrophs that rely on carbon substrates provided by their host to survive (Johns, 2014; Sally E Smith & F Andrew Smith, 2011). Indeed, plants transfer nearly 4-20% of the photosynthetically fixed carbon to the fungal partner (Jung et al., 2012) in the form of sugars (Bago et al., 2000) and lipids (Keymer & Gutjahr, 2018). In return, the fungi improve the supply of water and nutrients, such as phosphate and nitrogen, toward the host plant through extra- and intra-radical hyphae, arbuscules and the root apoplast

interface (Parniske, 2008). Several scientific papers have reported that plants inoculated with AMF exhibit more efficient water uptake and translocation of macro- and micronutrients to the shoot compared to non-mycorrhizal plants (Gamalero et al., 2004; Ortas et al., 2011; Rouphael et al., 2015; Smith & Read, 2008; Thirkell et al., 2017; Zhu et al., 2016). A plethora of studies has also investigated the effectiveness of AMF under abiotic stress conditions, such as drought (Asrar et al., 2012; Baum et al., 2015; Jayne & Quigley, 2014; Wu et al., 2013), salinity (Garg & Chandel, 2011; Latef & Chaoxing, 2011; Porcel et al., 2012), heavy metal soil contamination (de Andrade & da Silveira, 2008; Lee & George, 2005), and adverse pH conditions (Cardarelli et al., 2010; Rouphael et al., 2010). Besides the effect of AMF on plant nutrition and abiotic stress tolerance, a growing body of literature is showing the impact of AM symbiosis on plant immunity (Maffei et al., 2014; Nair et al., 2015; Ren et al., 2015; Shrivastava et al., 2015; Song et al., 2015).

Mycorrhizal fungi are able to enhance plant defense against a broad range of pathogens and pests, a phenomenon known as mycorrhizal-induced resistance [MIR; (Pozo & Azcón-Aguilar, 2007; Pozo et al., 2013; Rivero et al., 2021)]. In the frame of mycorrhizal-induced resistance, the fungi modulate plant defense signaling pathways, by generally upregulating the JA pathway, while suppressing the SA pathway in addition to interacting with various other plant hormones (Bucher et al., 2014; Cameron et al., 2013; Jung et al., 2012). In this way, mycorrhizal-induced resistance stimulates the plant immune system to a primed state that leads to a more efficient activation of defense responses upon exposure to biotic stress (Martinez-Medina et al., 2016; Mauch-Mani et al., 2017). In the absence of biotic stress, mycorrhizal plants exhibit slightly activated defenses, thus allowing plants to redirect resources to other biological functions (Jung et al., 2012; Pozo & Azcón-Aguilar, 2007). In the presence of biotic stress, mycorrhizal plants can trigger faster and stronger defenses both below- and aboveground (Rivero et al., 2021; Verhage et al., 2009). As it has been shown, mycorrhizal plants exhibit better survival rate, enhanced growth, and increased resistance against plant pathogens via competition for nutrients, space and photosynthates, rhizosphere alteration, and induction of host-plant defense (Dowarah et al., 2021). Several scientific papers have reported enhanced defense responses of mycorrhizal plants against a broad range of aboveground pathogens, including bacteria (Fiorilli et al., 2018; Malik et al., 2016; Tiénébo et al., 2019), fungi (Campo et al., 2020; Castellanos-Morales et al., 2011; Marquez et al., 2018; Nair et al., 2015; Sanmartín, Pastor, et al., 2020; Sanmartín, Sánchez-Bel, et al., 2020), and viruses (Maffei et al., 2014; Thiem et al., 2014).

AM fungi can also influence the quality and quantity of the resources available to herbivorous insects (Locke & Crawford, 2022). In this frame, AMF may increase herbivore performance by increasing the amount or quality of the host plant material available to them (Gange et al., 2005; Goverde et al., 2000; Hoffmann et al., 2009; Real-Santillán et al., 2019; Vannette & Hunter, 2013). Indeed, plants growing with AM fungi can be even 30% larger than plants without AMF (Gworgwor & Weber, 2003), and the AM-mediated increased quantity of resources can result in increased herbivore performance. In contrast, AMF can decrease herbivore performance by increasing the ability of their host plants to produce nutritionally expensive chemical defenses (Gange & West, 1994; Rivero et al., 2021; Song et al., 2013; Vannette & Hunter, 2013). For example, mycorrhiza are able to increase constitutive defenses against herbivores (Bennett et al., 2009; Formenti & Rasmann, 2019; Wang et al., 2015). In general, it has been proposed that the effects of mycorrhizal colonization under ecological settings are based on the feeding

behavior and the degree of specialization of the herbivorous insect (Gehring & Bennett, 2009; Koricheva et al., 2009). Thus, it has been proposed that mycorrhizal fungi generally have a negative effect on generalist leaf chewers and neutral or positive effect on specialist leaf chewers and phloem feeders (Pineda et al., 2013).

Trichoderma spp.

Trichoderma (teleomorph *Hypocrea*) is a genus of filamentous ascomycete that are among the most frequently isolated soil microorganisms (Druzhinina et al., 2011; Etschmann et al., 2015; Harman et al., 2004; Morán-Diez et al., 2015). The success of *Trichoderma* strains in the rhizosphere is due to their high reproductive capacity, ability to survive under unfavorable conditions, efficiency in the utilization of nutrients, capacity to modify the rhizosphere and compete with plant pathogenic fungi (Benítez et al., 2004; Harman, 2006). *Trichoderma* fungal strains are also able to colonize plant roots as avirulent symbionts (Harman et al., 2004). As it has been observed, plant-derived sucrose is an important source provided to the fungi by the host plant. In exchange, the fungi offer the plant a plethora of benefits. Among them are the promoted nutrient solubilization and absorption, improved yield, and increased tolerance to abiotic stresses. An array of scientific evidence has indicated that application of *Trichoderma* spp. in the rhizosphere improves plant morphology (H. Contreras-Cornejo et al., 2015; Contreras-Cornejo et al., 2009; Halifu et al., 2019; Yedidia et al., 2001) and physiology (Doni et al., 2014; Mishra & Salokhe, 2011; Shukla et al., 2012). Furthermore, roots of *Trichoderma*-colonized plants have exhibited a higher ability to explore soils and improve nutrient uptake (Altomare et al., 1999; Colla et al., 2015; Harman et al., 2004; Li et al., 2015). Plenty of studies have investigated the positive effect of *Trichoderma* strains on increasing the yield of several crops (El-Katatny & Idres, 2014; Haque et al., 2012; Idowu et al., 2016; Mahmood & Kataoka, 2018; Naznin et al., 2015). Moreover, *Trichoderma* strains have also exhibited the ability to alleviate the effects of abiotic stresses, such as salinity (Brotman et al., 2013; Contreras-Cornejo et al., 2014; Hashem et al., 2014; Mastouri et al., 2010, 2012; Qi & Zhao, 2013; Rawat et al., 2012; Zhang et al., 2016), drought (Contreras-Cornejo et al., 2009; H. A. Contreras-Cornejo et al., 2015; Shukla et al., 2012), heavy metal soil contamination (Rawat & Tewari, 2011), and adverse temperature (Ghorbanpour et al., 2018; Montero-Barrientos et al., 2010). Apart from the positive effects of *Trichoderma* strains on plant growth, nutrient uptake and abiotic stress alleviation, a plethora of studies has investigated the impact of *Trichoderma* spp. on plant immunity, mainly against pathogenic microbes (Alizadeh et al., 2013; Contreras-Cornejo et al., 2011; Djonovic et al., 2007; Gupta et al., 2014; Harman et al., 2004; Segarra et al., 2007).

Trichoderma strains have been broadly used to suppress plant diseases and the growth of pathogens in contact with plant tissues, or in terms of pathogen antagonism in the soil environment under both greenhouse and field conditions (Harman et al., 2004). *Trichoderma* spp. are known to raise resistance against pathogens by the induction of ISR in the plant (Segarra et al., 2009; Shoresh et al., 2005; Tucci et al., 2011). Nevertheless, the nature of *Trichoderma* spp.-induced ISR remains under discussion (Agostini et al., 2019). Several studies have indicated that some *Trichoderma* strains are able to elicit ISR in a JA- and ET-dependent manner in the

leaves of dicot plants (Contreras-Cornejo et al., 2011; Hermosa et al., 2012; Salas-Marina et al., 2011); whereas others have concluded that *Trichoderma* strains might trigger plant responses in a SA-dependent manner, mainly because of the upregulation of pathogenesis-related (PR) proteins (PR1, PR2 and PR5) (Hermosa et al., 2012; Mathys et al., 2012). Therefore, the *Trichoderma*-mediated induction of the JA, ET, or SA pathways within a single plant seems to indicate a rather intricate mechanism for resistance activation (Agostini et al., 2019).

Trichoderma fungi can also mediate plant-herbivore interactions through the activation of systemic plant defenses and the attraction of natural enemies of the herbivores (Poveda, 2021). Studies have shown that *Trichoderma* strains are capable of mounting plant resistance against arthropods belonging to the orders of Thysanoptera, Hemiptera and Heteroptera, by activating direct and indirect plant defense responses (Alınç et al., 2021; Battaglia et al., 2013; Coppola et al., 2017; Coppola, Cascone, et al., 2019; Coppola, Diretto, et al., 2019; Muvea et al., 2014). For instance, Muvea et al. (2014) showed that onion plants root-inoculated with *Trichoderma* demonstrated significantly lower feeding punctures by *Thrips tabaci*. Similarly, *Trichoderma* spp. have shown protective effects on plants against leaf-chewing herbivores through the modulation of direct and indirect defenses (Contreras-Cornejo, Del-Val, et al., 2018; Contreras-Cornejo, Macías-Rodríguez, et al., 2018; Rodríguez-González et al., 2018; Zhou et al., 2018). For example, the inoculation of roots with *T. atroviride* reduced the foliar damage caused by the leaf-chewing herbivore *Spodoptera frugiperda* (Contreras-Cornejo, Macías-Rodríguez, et al., 2018) in maize plants.

Justification, aims and model system

It is predicted that by 2050, the global population will reach approximately 9.1 billion people. In order to feed the increasing world population, a raise of about 70% in agricultural food production is required (Godfray et al., 2010). At the same time, biotic (i.e., pathogenic fungi and bacteria, insect herbivores, weeds, viruses) and abiotic (i.e., global warming, environmental pollution) stress factors are responsible for extensive crop yield losses and thus threaten food security. So far, the conventional agricultural methods followed to increase crop productivity and protect plants against biotic and abiotic stresses have resulted in serious environmental pollution and ecological damage. Therefore, the adoption of sustainable and environmentally friendly agricultural practices is a promising strategy to overcome these challenges and ensure high quantity and quality of yields in the coming years. In this frame, the interest in using beneficial microbes in agriculture has increased significantly due to the ability of these microbes to promote plant growth and productivity, improve plant resistance against pathogens and insect pests and ameliorate plant tolerance to abiotic stressors (Fernández-Lizarazo & Moreno-Fonseca, 2016; Lee Díaz et al., 2021; Wubs et al., 2019).

In the present Doctoral study, tomato (*Solanum lycopersicum*) was used as a model plant to investigate the effect of two beneficial root microbes- the AMF *Rhizophagus irregularis* and the growth-promoting fungus *Trichoderma harzianum*- on modulating its direct and indirect defenses against herbivory. Tomato belongs to the *Solanaceae* plant family (Al-Hilphy et al.,

2021) and is the second most cultivated vegetable crop throughout the world. It is also one of the most extensively consumed vegetables, and a rich source of bioactive chemicals (i.e., vitamin A and C, carotenoids and phenolics) with several advantages for human health (Domínguez et al., 2020; Lorenzo & Munekata, 2016; Vargas-Ramella et al., 2021; Zamuz et al., 2021). However, tomato is highly susceptible to numerous pests, which can severely affect its yield, nutritional value and taste (Zhang et al., 2021).

As herbivore pests, were used larvae of the leaf-chewing lepidopterans *Manduca sexta* L. and *Spodoptera exigua* (Hübner). *Manduca sexta* (Lepidoptera: Sphingidae), commonly known as the tobacco hornworm or Carolina sphinx moth, has been extensively used as a model system for insect biochemistry research, physiology, neurobiology, development, and immunity (Baldwin, 2001; Kanost et al., 2016; Riddiford et al., 2003; Shields & Hildebrand, 2001; Späthe et al., 2013). *M. sexta* larvae are oligophagous insect pests of the *Solanaceae* plant family (del Campo C & Renwick, 1999) -specialized in the nightshade (Yamamoto & Fraenkel, 1960)- therefore, can tolerate a substantial challenge from the defense metabolites synthesized by solanaceous plants (Kanost et al., 2016). *Spodoptera exigua* (Lepidoptera: Noctuidae), commonly known as the beet armyworm, is a polyphagous widely distributed insect, whose larvae feed on several crop species, such as cotton, cabbage, alfalfa, lettuce, and tomato (Moulton et al., 2000; Wang et al., 2006; Zheng et al., 2011). Despite, their low degree of specialization, *S. exigua* larvae can cause severe losses on tomato crop production (Taylor & Riley, 2008). At the same time, unlimited use of insecticides to control *S. exigua* populations has led to the evolution of its insecticide resistance (Delorme et al., 1988; Mascarenhas et al., 1998; Moulton et al., 2000). Therefore, there is a great need for novel, environmentally friendly approaches to protect tomato crops from *S. exigua*.

In the last decades, metabolomic analyses have provided novel insights in the metabolomic shifts that underlie the complex relationships of plants with herbivores and fungi (Peters et al., 2018; Salazar et al., 2018; Sardans et al., 2020; Sardans et al., 2011). In particular, the field of "Eco-metabolomics" (Kuzina et al., 2009; Peñuelas & Sardans, 2009) includes studies that aim at elucidating the responses, acclimatization, and adaptation of living organisms to adverse environmental conditions (Allevato et al., 2019; Rivas-Ubach et al., 2018; Rivas-Ubach et al., 2013). In the framework of my PhD study, I used an integrated "omics" approach, combining transcriptomics and metabolomics, with the aim to unravel the molecular and chemical mechanisms that underlie microbe-induced resistance against insect herbivores. Overall, this study aimed to explore the potential of root symbionts in protecting crop plants against insect herbivores. The results of this research most likely will contribute to the transformation of conventional to sustainable agriculture, characterized by a drastic reduction in the use of chemical insecticides in order to protect the environment and human health.

The results of my study are presented in three chapters, each with a specific hypothesis:

1. Root inoculation of tomato plants with the arbuscular mycorrhizal fungus *R. irregularis* and the growth-promoting fungus *T. harzianum* would affect the leaf metabolome and this effect would cascade up influencing the metabolome of the oligophagous insect herbivore *M. sexta*, leading to changes in its metamorphosis success. (Chapter 1).

2. Root inoculation with *R. irregularis* and *T. harzianum* would cause a wide re-arrangement in the transcriptome and metabolome of tomato leaves in response to *M. sexta* herbivory (Chapter 2).
3. Root inoculation of tomato plants with *R. irregularis* and *T. harzianum* would enhance plant indirect defense responses to *S. exigua*, by increasing the attraction of the omnivorous predator *Macrolophus* pygmaeus. This enhancement would be mediated by changes in the emission of HIPVs (Chapter 3).

Overview of the manuscripts

Manuscript 1

Title

Cascading effects of root microbial symbiosis on the development and metabolome of the insect herbivore *Manduca sexta* L.

Publication status

This manuscript has been published in the open access journal Metabolites, MDPI.

Bibliographical data

Papantoniou, D.; Vergara, F.; Weinhold, A.; Quijano, T.; Khakimov, B.; Pattison, D.I.; Bak, S.; van Dam, N.M.; Martínez-Medina, A. Cascading Effects of Root Microbial Symbiosis on the Development and Metabolome of the Insect Herbivore *Manduca sexta* L. *Metabolites* **2021**, *11*, 731. https://doi.org/10.3390/metabo11110731

Short summary

In thesis manuscript 1, it was shown that the beneficial root fungi, *R. irregularis* and *T. harzianum*, affected the metabolome of tomato shoots leading to enhanced levels of defense metabolites. This effect cascaded up on the metabolome of *M. sexta* herbivores fed on root-inoculated plants, thereby negatively affecting their development and metamorphosis. These findings point to the potential of using beneficial root microbes in order to sustainably protect crops against insect herbivores.

Manuscript 2

Title

Root colonization by the mutualistic fungi *Trichoderma harzianum* or *Rhizophagus irregularis* alters the transcriptomic and metabolomic defense response of tomato plants to *Manduca sexta* L. herbivory

Publication status

Thesis manuscript 2 is not published/ or submitted for publication. Parts of this chapter will be submitted for publication in the coming future after being enriched with more experimental data and analyses. The supplementary material related to the data presented in this chapter can be found in the following link or in the CD-ROM included in each thesis copy.

Short summary

In thesis manuscript 2, it was shown that the root mutualists, *R. irregularis* and *T. harzianum*, modulated tomato plant defenses in response to *M. sexta herbivory*, both on the transcriptomic and metabolomic level. Herbivory led to the upregulation of genes involved in stress-related responses. It is thus conceivable that root mutualists could serve as a promising tool in sustainable crop protection.

Manuscript 3

Title

Root symbionts alter herbivore-induced indirect defenses of tomato plants by enhancing predator attraction

Publication status

This manuscript has been published in the journal Frontiers in Physiology, special section Invertebrate Physiology.

Bibliographical data

Papantoniou D, Chang D, Martínez-Medina A, van Dam NM and Weinhold A (2022) Root symbionts alter herbivore-induced indirect defenses of tomato plants by enhancing predator attraction. *Front. Physiol.* 13:1003746. https://doi.org/10.3389/fphys.2022.1003746

Short summary

In thesis manuscript 3, it was shown that the root mutualists, *R. irregularis* and *T. harzianum*, are able to modulate the indirect defenses of tomato in response to *S. exigua* herbivory. The blend of volatiles emitted by microbe-inoculated herbivore infested plants was more attractive to the omnivorous mirid bug *M. pygmaeus*. These findings thus highlight the potential of employing root symbionts in Integrated Pest Management (IPM) applications.

Manuscript 1

FORM 1

Manuscript No. 1

Manuscript title: "Cascading effects of root microbial symbiosis on the development and metabolome of the insect herbivore *Manduca sexta* L."

Authors: Papantoniou Dimitra, Vergara Fredd, Weinhold Alexander, Quijano Teresa, Khakimov Bekzod, Pattison David Ian, Bak Søren, Martínez-Medina Ainhoa, van Dam Nicole

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The candidate is

☑ First author, ☐ Co-first author, ☐ Corresponding author, ☐ Co-author.

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Authors' contributions (in %) to the given categories of the publication

Author	Conceptual	Data analysis	Experimental	Writing the manuscript	Provision of material
Papantoniou Dimitra	33.3%	70%	80%	60%	60%
Vergara Fredd		15%			
Weinhold Alexander		15%			
Quijano Teresa			20%		20%
Martínez-Medina Ainhoa	33.3%			20%	20%
van Dam Nicole	33.3%			20%	
Total:	100%	100%	100%	100%	100%

metabolites

MDPI

Article

Cascading Effects of Root Microbial Symbiosis on the Development and Metabolome of the Insect Herbivore *Manduca sexta* L.

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Abstract: Root mutualistic microbes can modulate the production of plant secondary metabolites affecting plant–herbivore interactions. Still, the main mechanisms underlying the impact of root mutualists on herbivore performance remain ambiguous. In particular, little is known about how changes in the plant metabolome induced by root mutualists affect the insect metabolome and post-larval development. By using bioassays with tomato plants (*Solanum lycopersicum*), we analyzed the impact of the arbuscular mycorrhizal fungus *Rhizophagus irregularis* and the growth-promoting fungus *Trichoderma harzianum* on the plant interaction with the specialist insect herbivore *Manduca sexta*. We found that root colonization by the mutualistic microbes impaired insect development, including metamorphosis. By using untargeted metabolomics, we found that root colonization by the mutualistic microbes altered the secondary metabolism of tomato shoots, leading to enhanced levels of steroidal glycoalkaloids. Untargeted metabolomics further revealed that root colonization by the mutualists affected the metabolome of the herbivore, leading to an enhanced accumulation of steroidal glycoalkaloids and altered patterns of fatty acid amides and carnitine-derived metabolites. Our results indicate that the changes in the shoot metabolome triggered by root mutualistic microbes can cascade up altering the metabolome of the insects feeding on the colonized plants, thus affecting the insect development.

Keywords: arbuscular mycorrhizal fungi; *Trichoderma*; *Manduca sexta*; *Solanum lycopersicum*; LC–qToF–MS; metamorphosis

1. Introduction

Plants are a nutritious food source for insect species that belong to a wide range of taxonomic groups. To defend themselves against phytophagous insects, plants can produce a large range of metabolites that have repellent, anti-nutritive, or toxic effects on herbivores [1]. Commonly, they are secondary plant metabolites, such as alkaloids, benzoxazinoids, glucosinolates, terpenoids, phenolics, non-protein amino acids and cyanogenic glucosides [2,3]. Moreover, plants produce a number of defense proteins that reduce the digestibility of plants, such as protein inhibitors, α-amylase inhibitors, and polyphenol

oxidases [4]. These metabolites can be produced constitutively or are induced upon herbivore damage. Many of these metabolites are either toxic or deterrent to herbivores, thereby reducing herbivore damage. In addition, some metabolites can also disrupt molting and other developmental and physiological processes in insects with lethal consequences [5–7]. For instance, alkaloids can affect nerve signal transmission in insects by disturbing the cell membrane and the cytoskeletal structure, causing the collapse and leakage of cells [8]. In addition, terpenoids can disturb the nervous system of insects by inhibiting acetylcholinesterase [9]. Furthermore, they can inhibit ATP synthase, the alkylation of nucleophiles and interfere with insect molting [10]. Many herbivores have evolved mechanisms to overcome the negative effects of plant defense metabolites. These include, among others, the rapid excretion and detoxification through conjugation and breakdown [11]. Moreover, specialist herbivores can also sequester some defensive chemicals and use them for self-defense against their own natural enemies [12–14]. As a result, specialized herbivores feeding on defended plants can ingest plant-produced toxic metabolites without suffering major consequences. Remarkably, anti-herbivore metabolites that are affecting basic processes, such as molting, are less easy for insect herbivores to adapt to, and therefore can also affect specialists.

In addition to detrimental interactions, mutualistic plant interactions are also frequent in nature [15]. Plants nurture a vast community of mutualistic microbes in their root systems, which provides their host plants with essential functions related to nutrient acquisition and immune system modulation. Among the most abundant and widespread root associated mutualistic microbes are the arbuscular mycorrhizal (AM) and *Trichoderma* fungi, which establish root symbioses with the majority of land plants [16,17]. Mycorrhizal fungi have co-evolved with plants for 400 million years and form symbiotic interactions with ≈80% of all plant species [18–21]. Mycorrhizal roots form an extensive network of fungal hyphae, which increases the plant's exploratory capacity for water and mineral nutrients. Specialized fungal structures, called arbuscules, develop within root cells to facilitate nutrient exchange between the partners [20,21]. *Trichoderma* fungi are also ubiquitous soil microbes that display relatively low host specificity. In contrast to AM fungi, *Trichoderma* fungi are highly opportunistic, being capable of colonizing not only plant roots, but also aboveground plant parts and numerous other substrates such as wood and even other fungi [17,22–25].

Plants colonized by AM or *Trichoderma* fungi usually benefit from improved water and nutrient uptake, showing increased plant size, vigor, and nutrient levels [19,26–29]. These traits are important indicators of plant quality for herbivores [30–32]. In addition, root colonization by AM and *Trichoderma* fungi triggers a variety of molecular and biochemical responses in their host plants, including differential expression of defense-related genes and changes in the production of secondary metabolites. For instance, mycorrhizal colonization leads to a higher amount of iridoid glycosides and flavonoids in plantain and white clover leaves, respectively [33,34]. In addition, AM colonization has been shown to upregulate several plant secondary metabolite pathways, such as the carotenoid, the phenylpropanoid, and the antioxidant pathways [35,36], as well as to prime the accumulation of alkaloids and fatty acids upon herbivory [37]. Along the same lines, several studies have revealed that *Trichoderma* inoculation can trigger a higher accumulation of alkaloids, flavonoids, and phenolics; stress- and defense-related hormones; and transcripts related to different hormonal pathways in different plant species [38–43]. As a consequence, root colonization by AM or *Trichoderma* fungi can affect the performance of insect herbivores, as well as the amount of herbivore damage received by plants. However, the impact of the symbiotic fungi on herbivore performance can range from negative to positive, making the outcome of the symbiosis highly context-dependent [44].

Studies addressing the mechanistic basis of how plant mutualistic microbes affect plant–insect interactions focus specifically on physiological and metabolic changes in the plant. By contrast, how changes in the plant metabolome triggered by plant mutualistic microbes can affect the metabolome of insect herbivores, and thus affect their life

cycle, remains obscure. Here, we hypothesize that the impact of root colonization by AM and *Trichoderma* fungi on the plant metabolome can cascade up, thereby affecting the metabolome of insect herbivores that feed on the leaves of root-colonized plants. We further hypothesize that these changes in the herbivore's metabolome will affect its life cycle. By using tomato (*Solanum lycopersicum*) and the specialist chewing insect *Manduca sexta* as a model system, we found that root colonization by *Rhizophagus irregularis* or *Trichoderma harzianum* negatively affected the metamorphosis success of larvae that fed on the leaves of root-colonized plants. We further found that root colonization by the mutualistic fungi significantly affected the metabolome of the leaves as well as that of fat body and guts of larvae feeding on the colonized plants. Overall, our results indicate that the impact of root-mutualistic microbes can cascade up the trophic food chain, affecting the plant metabolome as well as the metabolism and the development of insect herbivores.

2. Results

2.1. Root Colonization by Rhizophagus irregularis or Trichoderma harzianum Impaired Pupation and Adult Emergence of Manduca sexta Larvae

We first aimed to assess whether root colonization by *R. irregularis* and *T. harzianum* affects the development of *M. sexta* larvae that fed on leaves of the root-inoculated plants. With this aim, we established a bioassay, in which we inoculated tomato roots with the mutualistic microbes and allowed *M. sexta* larvae to feed on the inoculated plants during the larval stage. After this, we assessed the impact of the different plant treatments on insect development, pupation, and adult emergence. We observed neither significant differences in the time required for the larvae to enter the pre-pupal stage, nor in the time required to complete the pupation process (Table 1). However, the number of individuals that successfully developed into normal pupae differed among the plant treatments. About 75% of the pupae developed by larvae fed on non-inoculated plants or *R. irregularis*-inoculated plants looked healthy (Table 1, Figure 1A). By contrast, about 60% of the larvae fed on *T. harzianum*-inoculated plants formed pupae with an aberrant morphology (Table 1, Figure 1A). Even though there were no significant differences in the pupal weight among the different plant treatments (Table 1), we observed significant differences in the sex ratio of the healthy pupae. Larvae fed on non-inoculated plants showed a female-based sex ratio of 0.69 (Table 1). The sex ratio of pupae emerging on *R. irregularis* plants was male-based (female: male ratio 0.3), being significantly different from that on control plants ($\chi^2 = 4.196$, $\alpha = 0.05$). On *T. harzianum*-inoculated plants, the sex ratio was male-based as well, but the low number of healthy pupae recovered was not sufficient to detect statistically significant differences (Table 1). Most of the healthy pupae that were formed by individuals reared on control plants eclosed, and the emerged adults presented a normal morphology (Table 1, Figure 1B). By contrast, fewer healthy adults emerged from the individuals reared on *R. irregularis* plants and a higher number of adults showed an anomalous morphology, mostly underdeveloped wings (Table 1, Figure 1B). The number of healthy pupae formed by individuals reared on *T. harzianum*-colonized plants was not enough for detecting differences (Table 1, Figure 1B). In summary, the performance of individuals varied among the different plant treatments. About 53% of the surviving larvae that were reared on non-inoculated plants developed into apparently healthy adults. This percentage was much lower for individuals reared on *R. irregularis*-(27%) or on *T. harzianum* (15%)-inoculated plants. Our results thus indicate that root colonization by the mutualistic microbes has a strong effect on the metamorphosis success of *M. sexta* larvae that had fed on the leaves of the root-inoculated plants.

Table 1. Impact of tomato root inoculation by *Rhizophagus irregularis* or *Trichoderma harzianum* on *Manduca sexta* pupation and adult emergence.

Treatment	No Larvae Reaching the Pre-Pupa Stage	Days Until Pre-Pupation (±SD)	Days until Pupation (±SD)	Total No of Pupae	No. of Pupae with Normal Morphology	No. of Pupae with Anomalous Morphology	Weight (g) of Normal Pupae (±SD)	F/M Ratio of the Normal Pupae	Number of Normal Pupae Not Eclosing	No of Moths with Anomalous Morphology	No of Moths with Normal Morphology
Non-inoculated	19	23.39 ± 1.47	22.06 ± 3.83	17	13	4	2.65 ± 0.49	9:4	2	1	10
R. irregularis	15	20.41 ± 2.54	19.94 ± 4.45	15	11	4	2.71 ± 0.61	3:8	3	4	4
T. harzianum	13	22.69 ± 1.9	11.61 ± 6.59	12	5	7	1.61 ± 0.91	2:3	1	2	2
One-way ANOVA	-	ns	ns	-	-	-	ns	$\chi^2 = 4.196$	-	-	-

One-way ANOVA evaluated the effect of the different treatments on the days required until pre-pupation, the days required for pupation, and the weight of the pupae showing a normal morphology. Pearson's chi-squared test evaluated the effect of the different treatments on the ratio of normally developed female to male *M. sexta* pupae. SD: standard deviation, ns: not significant.

Figure 1. The impact of root colonization by *Rhizophagus irregularis* or *Trichoderma harzianum* on the morphology of the pupae and moths developed by *Manduca sexta* larvae that had fed from root-inoculated tomato plants. (**A**) Representative pupae with anomalous morphology developed by larvae that had fed from *T. harzianum*-inoculated plants (right panel), and a representative pupa presenting normal morphology developed by larvae that had fed form non-inoculated plants (left panel). (**B**) Representative *Manduca sexta* moths showing anomalous morphology (underdeveloped wings) formed by larvae that had fed from *R. irregularis*-inoculated plants (right panel) and a representative moth presenting normal morphology developed by larvae that had fed form non-inoculated plants (left panel).

2.2. Root Colonization by Rhizophagus irregularis or Trichoderma harzianum Altered the Metabolomic Profile of Tomato Leaves

We next reasoned that the significant impact of the beneficial microbes on *M. sexta* pupation and adult emergence (Table 1) should be associated with changes in the leaf metabolome induced by the mutualistic microbes. To assess this, we performed an experiment, in which tomato plants were root-inoculated with *R. irregularis* or *T. harzianum*, and leaf challenged with *M. sexta* larvae. Three weeks after challenging the plants, we explored the impact of the mutualistic microbes and the herbivore on the constitutive and herbivore-induced leaf metabolome using liquid chromatography–quadrupole time of flight- mass spectrometry (LC-qToF-MS). The comparison between the *R. irregularis*-inoculated and non-inoculated plants showed that after three weeks of herbivory, the metabolomes of *R. irregularis*-inoculated and non-inoculated plants greatly overlapped (Figure 2A, dark blue dots vs. pink dots). However, in the absence of herbivory, the leaf metabolome of the *R. irregularis*-inoculated and non-inoculated plants clearly separated (Figure 2A, light blue dots vs. light green dots). Interestingly, the features that significantly contributed to the separation comprised alkaloids (identification level of confidence 1

according to the Metabolomics Standard Initiative (MSI) [45]). More precisely, the feature m/z = 578.4056, rt = 6.2 min was annotated as α-tomatine and showed its highest qToF intensity in the leaves of *R. irregularis*-inoculated plants (Figure S1A, Table S1). Similarly, the features m/z = 578.4051, rt = 6.0 min and m/z = 416.3524, rt = 6.0 min were annotated as α-tomatine isomers (level of confidence 1 according to MSI) and displayed their highest qToF intensity in the leaves of *R. irregularis*-inoculated plants (Figure S1B,C, Table S1). The feature m/z = 576.3898, rt = 5.5 min was annotated as an α-dehydrotomatine isomer (MSI level of confidence 2) and showed its highest qToF intensity in the leaf samples of the non-inoculated plants after herbivory (Figure S1D, Table S1). Moreover, the features m/z = 576.3896, rt = 5.6 min and m/z = 414.3369, rt = 5.6 min were annotated as α-dehydrotomatine isomers (MSI level of confidence 2) and displayed their highest qToF intensity in the leaves of *R. irregularis*-inoculated and non-inoculated plants under herbivory, but also in the leaves of *R. irregularis*-inoculated plants in the absence of herbivory (Figure S1E,F, Table S1).

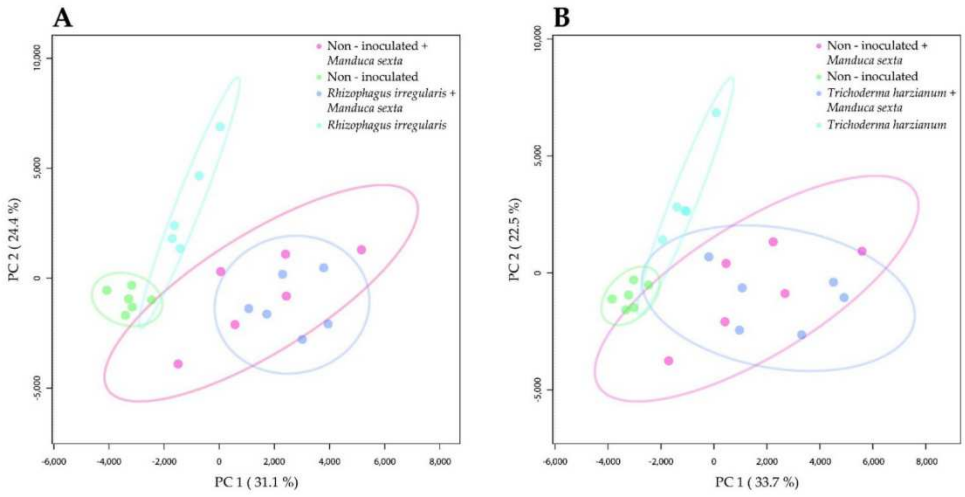

Figure 2. The impact of root colonization by *Rhizophagus irregularis* or *Trichoderma harzianum* and *Manduca sexta* shoot herbivory on the metabolome of tomato leaves. (**A**) Two-dimensional principal component analysis (PCA) scores plot of leaf samples of tomato plants that were non-inoculated or root-inoculated with *R. irregularis* and not challenged or shoot-challenged with *M. sexta* for three weeks. (**B**) PCA scores plot of leaf samples of tomato plants that were non-inoculated or root-inoculated with *T. harzianum* and not challenged or shoot-challenged with *M. sexta* for three weeks.

The comparison between the *T. harzianum*-inoculated and non-inoculated plants showed that three weeks after herbivory, the metabolomes of *T. harzianum*-inoculated and non-inoculated plants greatly overlapped (Figure 2B, dark blue dots vs. pink dots). In absence of herbivory, a clear separation was observed between the *T. harzianum*-inoculated and the non-inoculated plants (Figure 2B, light blue dots vs. light green dots). The separation of the different treatments was driven by features such as steroidal glycoalkaloids. The feature m/z = 578.4056, rt = 6.2 min (MSI level of confidence 1) was predicted as α-tomatine and showed its highest qToF intensity in the leaves of *T. harzianum*-inoculated plants (Figure S2A, Table S2). Likewise, the features m/z = 578.4051, rt = 6.0 min and m/z = 416.3524, rt = 6.0 min were annotated as α-tomatine isomers (MSI level of confidence 1) and displayed their highest qToF intensities in the leaves of the plants inoculated with *T. harzianum* (Figure S2B,C, Table S2). In addition, the feature m/z = 414.3369, rt = 5.6 min was annotated as an α-dehydrotomatine isomer (MSI level of confidence 2)

and showed the highest qToF intensity in the leaves of *T. harzianum*-inoculated plants (Figure S2D, Table S2). Another α-dehydrotomatine isomer, the feature m/z = 576.3896, rt = 5.6 min (MSI level of confidence 2) showed its highest qToF intensity in the leaves of non-inoculated and *T. harzianum*-inoculated plants after herbivory, but also in the leaves of *T. harzianum*-inoculated plants in the absence of herbivory (Figure S2E, Table S2).

Overall, our results indicate that root inoculation with *R. irregularis* or *T. harzianum* alters the shoot metabolome of tomato plants, although this effect seems to be overruled by a long period of *M. sexta* herbivory. Our results further indicate that steroidal glycoalkaloids are prominently present among the leaf metabolites showing the highest variation due to the root-microbial inoculation, and that they accumulated in a greater proportion in the leaves of the root-inoculated plants.

2.3. Root Colonization by Rhizophagus irregularis or Trichoderma harzianum Altered the Metabolic Profile of the Manduca sexta Larval Gut

We next aimed to understand whether the impact of the mutualistic microbes on the leaf metabolome (Figure 2) cascades up, thereby affecting the herbivore's metabolome. Therefore, we analyzed the metabolome of the caterpillars reared on the root-colonized plants by using ultra high performance liquid chromatography–mass spectrometry (UHPLC-MS). For conducting the analysis, we analyzed the metabolome of the gut and the fat body tissues separately.

When analyzing the gut tissue, we found that the metabolome of the larvae reared on non-inoculated plants did not separate from the metabolome of the larvae reared on *R. irregularis*-inoculated plants (Figure 3A). Still, we found several features, whose intensity significantly differed between the treatments. Among the annotated features that accumulated to a higher level in the gut tissue of larvae fed on non-inoculated plants, we found acyl-carnitines (m/z = 394.2951, rt = 12 min; m/z = 392.3157, rt = 17.9 min; and m/z = 378.3012, rt = 17.4) and glutathione (m/z = 308.0908, rt =1.1 min) (Figure 3B, Table 2; feature IDs 1, 3, 4, and 2, respectively; level of confidence of 3 for the acyl-carnitines and 2 for glutathione according to MSI). Among the annotated features that were indicated as significantly increased for the *R. irregularis*-inoculated plants, the amine piperidine (m/z = 86.0967, rt = 0.5 min, MSI level of confidence 2; Figure 3B, Table 2; feature ID 6) was found. In addition, the steroidal glycoalkaloid α-tomatine (m/z = 578.4056, rt = 8.4 min) was also annotated and was accumulated to higher levels in the gut tissue of larvae fed on *R. irregularis*-inoculated plants ($p < 0.001$) (Figure S3A; MSI level of confidence 1).

The comparison of the gut metabolome between larvae fed on non-inoculated and *T. harzianum*-inoculated plants revealed a clear separation between the gut tissues (Figure 4A). The predicted features that were shown as significantly increased for the non-inoculated samples included compounds such as the acyl-carnitines, more precisely linoleyl-carnitine (m/z = 424.3421, rt = 13.9 min) and γ-linolenoyl-carnitine (m/z = 422.3274, rt = 13.2 min; MSI level of confidence 2) (Figure 4B, Table 3; feature IDs 2 and 5, respectively). Among the predicted features that were accumulated to a significantly higher level in the *T. harzianum*-inoculated samples, the acyl-carnitine *O*-palmitoyl-carnitine (m/z = 400.3421, rt = 14.8 min) was annotated (Figure 4B, Table 3; MSI level of confidence 2; feature ID 14). Moreover, α-tomatine (m/z = 578.4049, rt = 8.4 min) was also annotated and was indicated as significantly more highly accumulated (p <0.001) in the gut of *T. harzianum*-inoculated larvae (Figure S4A; MSI level of confidence 1).

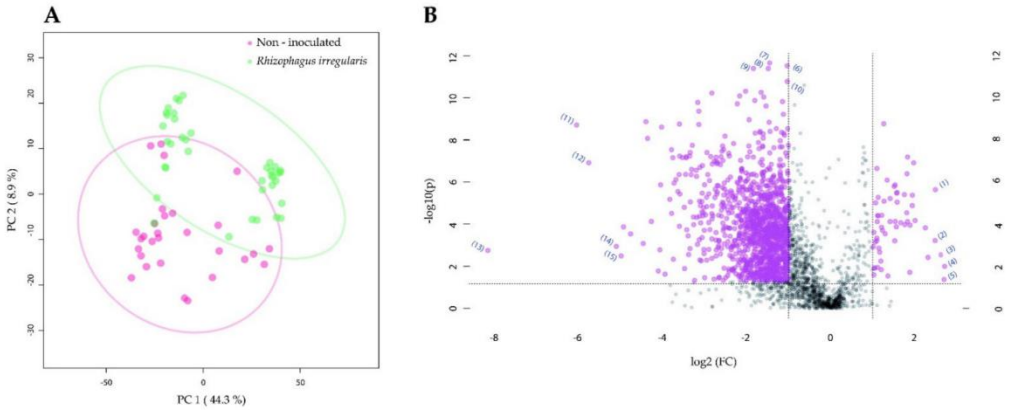

Figure 3. The impact of tomato root colonization by *Rhizophagus irregularis* on the gut metabolome of *Manduca sexta* larvae. (**A**) Two-dimensional principal component analysis (PCA) scores plot of *M. sexta* gut samples. (**B**) Significant metabolomic features annotated for the *M. sexta* gut samples. *Manduca sexta larvae* were fed for three weeks on leaves of tomato plants that were non-inoculated or root-inoculated with *R. irregularis*. In (**B**), the volcano plot displays fold change threshold (*x*-axis) of 2 and *t*-test threshold (*y*-axis) of 0.05. The purple circles represent features above the threshold. Both fold changes and *p*-values are log-transformed. The metabolomic features that showed the highest increase for the larvae reared on non-inoculated plants are found towards the right side of the volcano plot. The metabolomic features that showed the highest increase for the larvae reared on *R. irregularis*-inoculated plants are found towards the left side of the volcano plot. The most statistically significant metabolomic features for both treatments are found towards the top of the volcano plot.

Table 2. The most significant metabolomic features annotated for the gut samples of *Manduca sexta* larvae fed for three weeks on leaves of tomato plants non-inoculated and root-inoculated with *Rhizophagus irregularis*. The feature number corresponds to the features given in Figure 3B, which are given by their corresponding mass to charge ratio (m/z) and retention time (rt) in minutes. The fold change (FC) refers to the feature's qToF intensity and was calculated on the basis of a volcano plot conducted for the non-inoculated and *R. irregularis*-inoculated larvae. The volcano plot was conducted with a fold change threshold (*x*-axis) of 2 and *t*-test threshold (*y*-axis) of 0.05. The level of confidence of the identification/annotation is given according to Malinowska and Viant [45].

Feature ID	*Annotation*	*m/z*	*rt*	*FC*	*log_2(FC)*	*Raw p-Value*	*Level of Confidence*
1	$C_{23}H_{39}NO_4$	394.2951	12.0	5.63	2.5	$p < 0.0001$	3
2	$C_{10}H_{17}N_3O_6S$ (Glutathione)	308.0908	1.1	5.61	2.49	$p = 0.0006$	2
3	$C_{24}H_{41}NO_3$	392.3157	17.9	6.25	2.64	$p = 0.002$	3
4	$C_{23}H_{39}NO_3$	378.3012	17.4	6.56	2.71	$p = 0.01$	3
5	$C_{27}H_{39}O_3$	426.3009	17.9	6.49	2.7	$p = 0.04$	3
6	$C_5H_{11}N$ (Piperidine)	86.0967	0.5	0.49	−1.03	$p < 0.0001$	2
7	$C_{55}H_{94}N_{10}O_{16}P_2$	623.3105	8.0	0.37	−1.45	$p < 0.0001$	3
8	$C_{52}H_{97}N_8O_{17}P_3S$	616.3028	8.0	0.36	−1.48	$p < 0.0001$	3
9	No formula	110.0653	1.2	0.28	−1.84	$p < 0.0001$	4
10	No formula	132.1018	0.4	0.49	−1.03	$p < 0.0001$	4
11	$C_{12}H_{37}N_7OS$	328.2841	13.1	0.02	−6.05	$p < 0.0001$	3
12	$C_{23}H_{43}N_3O_6P_2$	520.2708	14.9	0.02	−5.76	$p < 0.0001$	3
13	$C_{14}H_{18}N_2O_5$	295.1293	4.9	0.004	−8.16	$p = 0.002$	3
14	No formula	782.0187	8.2	0.03	−5.1	$p = 0.001$	4
15	No formula	644.2370	10.4	0.03	−4.5	$p = 0.003$	4

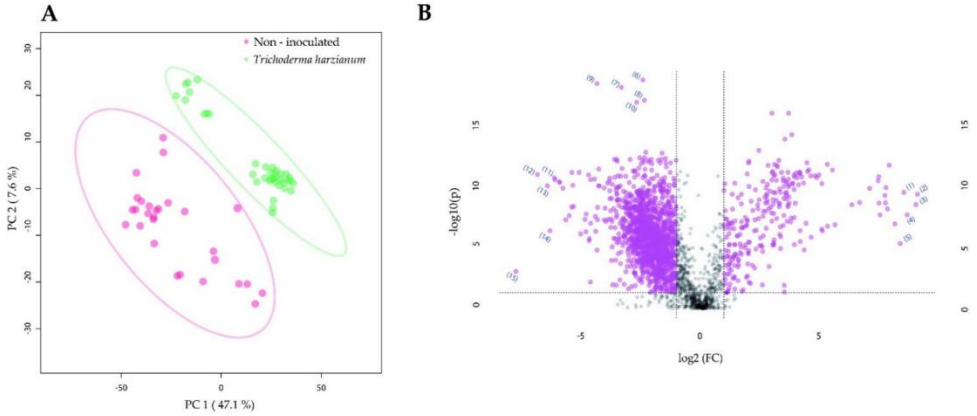

Figure 4. The impact of tomato root colonization by *Trichoderma harzianum* on the gut metabolome of *Manduca sexta* larvae. (**A**) Two-dimensional principal component analysis (PCA) scores plot of *M. sexta* gut samples. (**B**) Significant metabolomic features annotated for the *M. sexta* gut samples. *Manduca sexta* larvae were fed for three weeks on leaves of tomato plants that were non-inoculated or root inoculated with *T. harzianum*. In (**B**), the volcano plot displays fold change threshold (*x*-axis) of 2 and *t*-test threshold (*y*-axis) of 0.05. The purple circles represent features above the threshold. Both fold changes and *p*-values are log-transformed. The metabolomic features that showed the highest increase for the larvae reared on non-inoculated plants are found towards the right side of the volcano plot. The metabolomic features that showed the highest increase for the larvae reared on *T. harzianum*-inoculated plants are found towards the left side of the volcano plot. The most statistically significant metabolomic features for both treatments are found towards the top of the volcano plot.

Table 3. The most significant metabolomic features annotated for the gut samples of Manduca sexta larvae fed for three weeks on leaves of tomato plants that were non-inoculated and root-inoculated with Trichoderma harzianum. The feature number corresponds to the features given in Figure 4B and are given by their corresponding mass to charge ratio (m/z) and retention time (rt) in minutes. The fold change (FC) refers to the feature's qToF intensity and was calculated on the basis of a volcano plot conducted for the non-inoculated and T. harzianum-inoculated larvae. The volcano plot was conducted with a fold change threshold (*x*-axis) of 2 and *t*-test threshold (*y*-axis) of 0.05. The level of confidence of the identicication/annotation is given according to Malinowska and Viant [45].

Feature ID	Annotation	*m/z*	rt	FC	$\log_2$(FC)	Raw *p*-Value	Level of Confidence
1	$C_{23}H_{39}NO_4$	394.2951	12.0	378.82	8.57	$p < 0.0001$	3
2	$C_{25}H_{45}NO_4$ (linoleyl-carnitine)	424.3421	13.9	597.13	9.22	$p < 0.0001$	2
3	$C_{22}H_{42}N_2O$	351.3370	12.9	614.71	9.26	$p < 0.0001$	3
4	No formula	224.2010	9.8	388.27	8.6	$p < 0.0001$	4
5	$C_{25}H_{43}NO_4$ (γ-linolenoyl-carnitine)	422.3274	13.2	340.61	8.41	$p < 0.0001$	2
6	$C_{23}H_{42}NO_7P$	476.2772	13.0	0.19	−2.4	$p < 0.0001$	3
7	$C_{13}H_{16}N_4$	229.1444	7.9	0.1	−3.31	$p < 0.0001$	3
8	No formula	110.0653	1.2	0.2	−2.34	$p < 0.0001$	4
9	$C_8H_9NO_2$	152.0709	6.7	0.05	−4.34	$p < 0.0001$	3
10	$C_{10}H_9NO_4$	225.0872	1.3	0.16	−2.67	$p < 0.0001$	3
11	No formula	133.0866	7.9	0.01	−6.13	$p < 0.0001$	4
12	No formula	812.0668	8.0	0.009	−6.84	$p < 0.0001$	4
13	$C_{12}H_{37}N_7OS$	328.2841	13.1	0.01	−6.42	$p < 0.0001$	3
14	$C_{23}H_{45}NO_4$ (O-palmitoyl-carnitine)	400.3421	14.8	0.01	−6.31	$p < 0.0001$	2
15	$C_{14}H_{18}N_2O_5$	295.1293	4.9	0.005	−7.74	0.0001	3

2.4. Root Colonization by Rhizophagus irregularis or Trichoderma harzianum Altered the Metabolic Profile of the Manduca sexta Larval Fat Body

We found that there was no clear separation between the fat body metabolome of *M. sexta* larvae reared on non-inoculated plants and that of larvae reared on *R. irregularis*-inoculated plants (Figure 5A). However, among the features that were found to accu-

mulate to higher levels in the *R. irregularis*-inoculated samples, the compound (2*E*)-hexadecenoylcarnitine was annotated (m/z = 398.3265, rt = 13.7 min) (Figure 5B, Table 4; MSI level of confidence 2; feature ID 10). The steroidal glycoalkaloid alpha-tomatine (m/z = 578.4047, rt = 8.4 min) was also annotated, displaying higher levels of accumulation in the *R. irregularis*-inoculated larvae ($p < 0.0001$) (Figure S3B; MSI level of confidence 1).

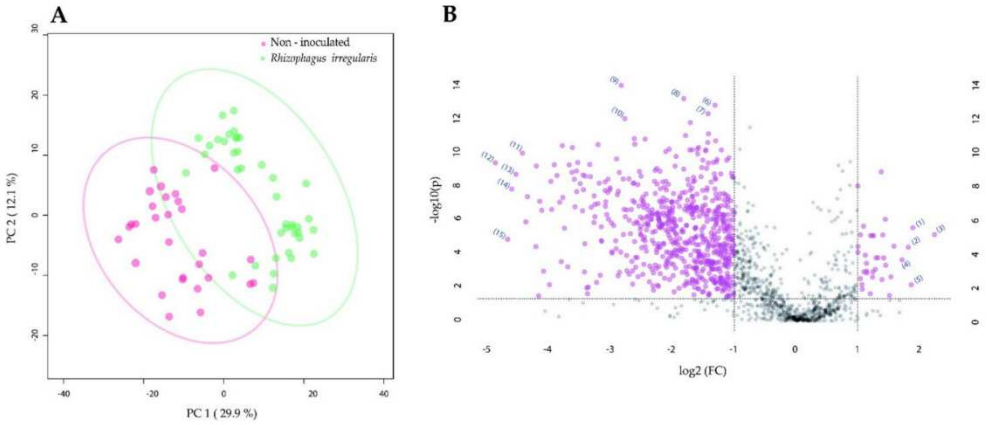

Figure 5. The impact of tomato root colonization by *Rhizophagus irregularis* on the fat body metabolome of *Manduca sexta* larvae. (**A**) Two-dimensional principal component analysis (PCA) scores plot of *M. sexta* fat body samples. (**B**) Significant metabolomic features annotated for the *M. sexta* fat body samples. *Manduca sexta* larvae were fed for three weeks on leaves of tomato plants that were non-inoculated, or root-inoculated with *R. irregularis*. In (**B**), the volcano plot displays fold change threshold (x-axis) of 2 and t-test threshold (y-axis) of 0.05. The purple circles represent features above the threshold. Both fold changes and p-values are log-transformed. The metabolomic features that showed the highest increase for the larvae reared on non-inoculated plants are found towards the right side of the volcano plot. The metabolomic features that showed the highest increase for the larvae reared on *R. irregularis*-inoculated plants are found towards the left side of the volcano plot. The most statistically significant metabolomic features for both treatments are found towards the top of the volcano plot.

Table 4. The most significant metabolomic features annotated for the fat body samples of *Manduca sexta* larvae fed for three weeks on leaves of tomato plants that were non-inoculated and root-inoculated with *Rhizophagus irregularis*. The feature number corresponds to the features given in Figure 5B, which are given by their corresponding mass to charge ratio (m/z) and retention time (rt) in minutes. The fold change (FC) refers to the feature's qToF intensity and was calculated on the basis of a volcano plot conducted for the non-inoculated and *R. irregularis*-inoculated larvae. The volcano plot was conducted with a fold change threshold (x-axis) of 2 and t-test threshold (y-axis) of 0.05. The level of confidence of the identification/annotation is given according to Malinowska and Viant [45].

Feature ID	*Annotation*	*m/z*	*rt*	*FC*	*log_2(FC)*	*p-Value*	*Level of Confidence*
1	$C_{25}H_{43}NO_5$	438.3210	11.9	3.71	1.89	$p < 0.0001$	3
2	$C_{32}H_{66}N_4O_7P_2S$	357.2138	6.8	3.52	1.82	$p < 0.0001$	3
3	$C_{10}H_{12}N_2O_3$	209.0909	0.9	4.73	2.24	$p < 0.0001$	3
4	$C_{37}H_{62}N_6$	591.5095	15.1	3.29	1.72	$p < 0.0001$	3
5	$C_{36}H_{74}N_2O_2$	567.5823	19.9	3.64	1.87	$p < 0.008$	3
6	$C_{23}H_4NO_7P$	476.2774	11.7	0.4	−1.31	$p < 0.0001$	3
7	No formula	175.1163	1.1	0.37	−1.42	$p < 0.0001$	4
8	No formula	112.0502	1.0	0.28	−1.82	$p < 0.0001$	4
9	No formula	671.3542	5.9	0.14	−2.83	$p < 0.0001$	4
10	$C_{23}H_{43}NO_4$ (2E)-hexadecenoylcarnitine)	398.3265	13.7	0.15	−2.77	$p < 0.0001$	2
11	No formula	224.2010	10.0	0.05	−4.42	$p < 0.0001$	4
12	$C_{37}H_{66}N_8O_{10}$	392.2516	6.9	0.03	−4.86	$p < 0.0001$	3
13	$C_{50}H_{94}N_8O_{17}$	360.5650	6.2	0.04	−4.53	$p < 0.0001$	3
14	$C_{41}H_{79}N_{14}O_5PS$	304.5352	5.9	0.04	−4.6	$p < 0.0001$	3
15	$C_{12}H_{37}N_7OS$	328.2839	13.1	0.04	−4.66	$p < 0.0001$	3

The analysis of the fat body metabolome of the *M. sexta* larvae revealed a clear separation between the fat body metabolome of the larvae reared on non-inoculated plants and the metabolome of the larvae reared on *T. harzianum*-inoculated plants (Figure 6A). Among the features that were indicated as significantly higher accumulated for the non-inoculated samples, the compound octadecanamide (m/z = 284.2950, rt = 19.6 min; MSI level of confidence 2; feature ID 4; Figure 6B, Table 5) was annotated. In the *T. harzianum*-inoculated samples, the metabolomic features annotated as octanoic acid (m/z =145.1222, rt = 8.0 min) and benzamide (m/z = 625.2662, rt = 15.9 min) were accumulated to a higher level (Figure 6B, Table 5; MSI level of confidence 2; feature IDs 12 and 14, respectively). In addition, the steroidal glycoalkaloid α-tomatine (m/z = 578.4047, rt = 8.4 min) was annotated and accumulated in significantly higher levels in the fat body of *T. harzianum*-inoculated larvae ($p < 0.0001$; Figure S4B; MSI level of confidence 1).

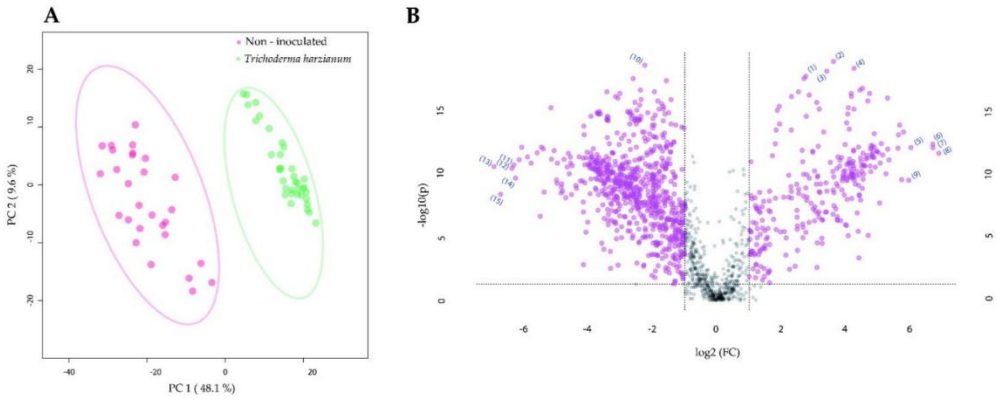

Figure 6. The impact of tomato root colonization by *Trichoderma harzianum* on the fat body metabolome of *Manduca sexta* larvae. (**A**) Two-dimensional principal component analysis (PCA) scores plot of *M. sexta* fat body samples. (**B**) Significant metabolomic features annotated for the *M. sexta* fat body samples. *Manduca sexta* larvae were fed for three weeks on leaves of tomato plants that were non-inoculated or root-inoculated with *T. harzianum*. In (**B**), the volcano plot displays fold change threshold (*x*-axis) of 2 and *t*-test threshold (*y*-axis) of 0.05. The purple circles represent features above the threshold. Both fold changes and *p*-values are log-transformed. The metabolomic features that showed the highest increase for the larvae reared on non-inoculated plants are found towards the right side of the volcano plot. The metabolomic features that showed the highest increase for the larvae reared on *T. harzianum*-inoculated plants are found towards the left side of the volcano plot. The most statistically significant metabolomic features for both treatments are found towards the top of the volcano plot.

Table 5. The most significant metabolomic features annotated for the fat body samples from *Manduca sexta* larvae fed for three weeks on leaves of tomato plants that were non-inoculated and root-inoculated with *Trichoderma harzianum*. The feature number corresponds to the features given in Figure 6B, which are given by their corresponding mass to charge ratio (m/z) and retention time (rt) in minutes. The fold change (FC) refers to the feature's qToF intensity and was calculated on the basis of a volcano plot conducted for the non-inoculated and *T. harzianum*-inoculated larvae. The volcano plot was conducted with a fold change threshold (x-axis) of 2 and t-test threshold (y-axis) of 0.05. The level of confidence of the identification/annotation is given according to Malinowska and Viant [45].

Feature ID	*Annotation*	*m/z*	*rt*	*FC*	*log2(FC)*	*p-Value*	*Level of Confidence*
1	$C_9H_{18}O_2$	159.1377	12.9	6.8	2.77	$p < 0.0001$	3
2	No formula	320.2558	14.5	12.36	3.63	$p < 0.0001$	4
3	$C_{17}H_{31}NO$	266.2483	13.6	10.71	3.42	$p < 0.0001$	3
4	$C_{18}H_{37}NO$ (octadecanamide)	284.295	19.6	19.24	4.27	$p < 0.0001$	2
5	$C_{36}H_{70}N_2O_4$	595.5408	15.8	65.57	6.04	$p < 0.0001$	3
6	$C_{27}H_{50}N_2O_2$	435.3944	17.8	105.25	6.72	$p < 0.0001$	3
7	$C_{36}H_{70}N_2O_6$	627.5310	14.9	105.4	6.72	$p < 0.0001$	3
8	$C_{28}H_{50}N_4O_8S$	603.3416	10.4	119.75	6.9	$p < 0.0001$	3
9	$C_{15}H_{41}N_6O_3PS$	417.2771	10.6	62.57	5.97	$p < 0.0001$	3
10	$C_{23}H_{42}NO_7P$	476.2774	11.7	0.21	−2.24	$p < 0.0001$	3
11	$C_{44}H_{74}N_5O_{10}P$	432.7648	8.0	0.01	−6.32	$p < 0.0001$	3
12	$C_8H_{16}O_2$ (octanoic acid)	145.1222	8.0	0.01	−6.37	$p < 0.0001$	2
13	$C_{58}H_{77}N_{31}O_7$	660.8389	8.0	0.008	−6.93	$p < 0.0001$	3
14	$C_{35}H_{36}N_4O_7$ (benzamide)	625.2662	15.9	0.01	−6.26	$p < 0.0001$	2
15	No formula	742.0044	7.87	0.009	−6.73	$p < 0.0001$	4

3. Discussion

Root mutualistic microbes such as mycorrhiza and *Trichoderma* fungi can modulate the production of plant secondary metabolites and thus affect the performance of the herbivores feeding on the colonized plants [37,43]. Here, we demonstrate that the changes in plant metabolomes induced by root colonizing mutualistic microbes cascade up and modulate the metabolome of the insect herbivores feeding on leaves of root-colonized plants. Our results further indicate that these changes in the insect metabolome can affect the ability of the insect to successfully complete metamorphosis. Indeed, we first observed that root colonization by the mutualistic microbes *R. irregularis* or *T. harzianum* leads to the impairment of important stages of the insect metamorphosis process, particularly pupation and adult emergence. Specifically, we found that most of the larvae reared on *T. harzianum*-inoculated plants developed into abnormal pupae and displayed higher mortality rates. We further found that most of the larvae reared on *R. irregularis*-inoculated plants developed into moths with anomalous morphologies and did not emerge. Taking into consideration that healthy *M. sexta* adult females can lay over 200 eggs [46], the impact of the mutualistic microbes on the metamorphosis success is expected to strongly decrease population growth rates. Moreover, we found that herbivores feeding on *R. irregularis*-colonized plants showed a shift towards a male-biased sex ratio. The sex ratio of lepidopteran insects influences their reproductive potential [47]. In the case of *M. sexta*, it has been reported that the optimum sex ratio (female to male) that ensures high levels of oviposition, fecundity, and fertility lies between 0.5 and 0.67 [48]. We found that the sex ratio of insects feeding on non-inoculated plants was very similar to the optimal range (0.69), whereas the sex ratio of insects that fed on *R. irregularis*-colonized plants was much lower (0.3), thereby reducing the reproductive potential of the colony. These results may further indicate that female *M. sexta* individuals are more sensitive to the shoot alterations triggered by root colonizing microbes, compared to the male individuals. Accordingly, it has been reported that female herbivores are more sensitive to environmental disturbance and host quality compared to male individuals [48,49]. Overall, these results indicate that the impact of beneficial microbes on the performance of insect herbivores goes beyond the larval stage and can affect further stages of the herbivore life cycle, potentially having a strong impact on insect population dynamics.

It has been reported that the cumulative physiological effects experienced by insect herbivores during the larval feeding stages can impair the pupae formation, moths' emergence, and even sex ratios [48,50,51]. In line with previous studies, our data indicated that root colonization by beneficial microbes altered the metabolome of the shoot of tomato plants [37,43,52]. Interestingly, we found several steroidal glycoalkaloids among the different features contributing strongly to the separation of leaf metabolomes of root-inoculated from non-inoculated plants. The abundance of steroidal glycoalkaloids was higher in leaves of plants inoculated with the mutualistic microbes. Steroidal glycoalkaloids in *Solanum* species function as prominent defense metabolites against herbivorous insects [53–56]. There are many reports demonstrating their toxicity and deterrence towards a wide range of herbivores [57–61]. Remarkably, besides affecting feeding and growth, steroidal alkaloids can affect other crucial physiological processes and interfere with insect development. It was suggested that steroidal glycoalkaloids might interact with the endocrine system of pests by interfering with hormone activity [62–64]. This is likely due to the fact that steroidal glycoalkaloids are derived from sterols and have similar chemical structures as the insect hormone ecdysone [65,66]. Hence, steroidal glycoalkaloids, through their similarity to sterols, are proposed to affect insect molting and metamorphosis, both processes that are regulated by ecdysone.

In addition to the strong effects of root colonization on herbivore development, we found an accumulation of the steroidal glycoalkaloid α-tomatine in the gut and fat body of herbivores reared on microbe-inoculated plants. It has been described that *M. sexta* has the ability to detoxify alkaloids, such as nicotine, most likely by degradation and excretion [67,68]. Typically, nicotine and many other alkaloids are absorbed by the midgut, pass into the haemolymph, are reabsorbed by the Malpighian tubules, and are finally excreted with the feces. Depending on the type of alkaloid, between 30 and 83% of them may be metabolized after absorption [68,69]. Although it could be that *M. sexta* is also able to degrade α-tomatine to some extent, our data indicated that the levels of α-tomatine in the gut and fat body of *M. sexta* were higher when the larvae were reared on plants that were colonized by the mutualistic microbes. Besides steroidal glycoalkaloids, we found a higher accumulation of piperidine in the gut of the larvae reared on *R. irregularis*-inoculated plants. Piperidine-containing alkaloids are complex structures that are derived from the amino acid *L*-lysine [70] and are able to disrupt the central nervous system of insects and mammals. For example, the piperidine alkaloids synthesized by the leguminous plant *Prosopis juliflora* have been reported to act as acetylcholinesterase inhibitors, butyrylcholinesterase inhibitors with Ca^{2+} channel blocking activity [71]. It is thus conceivable that enhanced levels of steroidal glycoalkaloids and piperidine alkaloids found in the insects that fed on the microbe-colonized plants might underlie, at least partially, the significant impact of the mutualistic microbes on *M. sexta* metamorphosis success.

Besides alkaloid-related metabolites, we found a higher accumulation of the metabolite benzamide in the fat bodies of larvae reared on *T. harzianum*-inoculated plants. Benzamides are amide derivatives of benzoic acid widely used as pesticides [72]. Treatment with benzamide chemicals has been related to an enhanced tissue lipid peroxidation and oxidative damage in juvenile catfish [73] and deformities in zebra fish embryos [74]. Our results thus suggest enhanced oxidative stress in the insects fed on *Trichoderma*-inoculated plants. Interestingly, we found a reduced accumulation of the metabolite glutathione in the gut of *M. sexta* larvae fed on microbe-inoculated plants. The primary role of glutathione (GSH) is to detoxify potentially deleterious substances and maintain the redox state in cells [75]. Insect glutathione S-transferases (GSTs) hold a pivotal role in detoxification and cellular antioxidant defense against oxidative stress by conjugating GSH to the electrophilic centers of natural and synthetic exogenous xenobiotics, including insecticides and allelochemicals [76,77]. Therefore, a reduced accumulation of glutathione in *M. sexta* larvae fed from microbe-inoculated plants might also reflect an enhanced oxidative stress, and thus a decreased fitness of the insects fed from the microbe-inoculated plants.

Our analyses further indicated that root colonization by the mutualistic microbes significantly altered the pattern of fatty acid amides in the gut and fat body of the insect. Although further analyses including standards would be required to unequivocally identify the altered metabolites, several studies indicate that fatty acid amides in the regurgitant of different species of lepidopteran larvae are important elicitors of plant defense responses [78–81]. The synthesis of fatty acid amides occurs by membrane-bound enzymes in the foregut and anterior midgut of the insect, utilizing insect-derived amino acids and host plant-derived fatty acids [78,82,83]. Even though it was demonstrated that they may play a complex role in nitrogen assimilation in insects [79], their exact physiological roles remain obscure [84]. Remarkably, we found that the root mutualists further triggered changes in insect fatty acids and lipids. For instance, we found a higher accumulation of octanoic acid in the fat body of larvae fed from *Trichoderma*-inoculated plants. Lipids are essential parts of cell and organelle membranes of insects; serve as important sources of energy; and act as precursors for secondary metabolites, waxes, pheromones, and defensive secretions [85–87]. Moreover, the metamorphosis process in *Sarcophaga argyrostoma* has been associated with specific changes in the profile of fatty acids, including octanoic acid [88]. Although further analysis would be required to establish solid conclusions, the changes in the fatty acid profile of the larvae triggered by the root-mutualistic fungi have the potential for altering the metamorphosis process of *M. sexta*.

In addition, we were able to predict the metabolite linoleyl-carnitine and other carnitine-derived metabolites in the gut and fat body of *M. sexta* larvae. It has been reported that carnitine-derived metabolites such as *L*-carnitine can affect the metabolism of lipids, proteins, and carbohydrates in mammals and zebrafish [89–91]. Moreover, carnitine and its acyl derivatives, which are involved in the lipid β-oxidation process, have been involved in the behavioral transition from the gregarious to solitary phase in locusts, possibly through modulating lipid metabolism and influencing the nervous system of the herbivore [92]. Although the ecological consequences of the altered levels of carnitine-derivatives in the body of *M. sexta* larvae may be different than in locusts, our results point to an important effect of root colonization by the mutualistic microbes on lipid metabolism.

In general, our study demonstrates that root colonization by the mutualistic microbes *R. irregularis* and *T. harzianum* impacts the metabolome of tomato shoots. These metabolomic alterations, triggered by the mutualistic microbes, affect the metabolome of *M. sexta* larvae feeding on the shoots of root-colonized plants. Among the most significant alterations, we found that *M. sexta* larvae feeding on the leaves of the root-colonized plants contain higher levels of the prominent steroidal glycoalkaloid α-tomatine. Moreover, feeding from microbial-colonized plants also led to changes related to oxidative damage, fatty acid metabolism, and lipid metabolism in the insect. Although further studies would be required to pinpoint the exact mechanism, we speculate that the microbe-triggered alterations in the insect metabolome underlie, at least partially, the impact of the root mutualists on insect metamorphosis.

4. Materials and Methods

4.1. Plant, Fungal, and Insect Material

Tomato (*Solanum lycopersicum*) cultivar Moneymaker was used in all the bioassays. Tomato seeds were obtained from Intratuin B.V (Woerden, the Netherlands). We used *Rhizophagus irregularis* as the AM fungus. The *R. irregularis* solid inoculum (INOQ-Sprint) was purchased from INOQ GmbH, Schnega, Germany (Available online: https://inoq.de/mykorrhiza-produkte/, accessed on 5 May 2021) with 220 mycorrhizal units per milliliter in sand. *Trichoderma harzianum* isolate T-78 (CECT 20714, Spanish Type Culture Collection) was maintained on potato dextrose agar (PDA, Sifin Diagnostics, Berlin, Germany) plates and regularly sub-cultured. The *T. harzianum* inoculum was prepared on a solid medium containing commercial oat and vermiculite [93]. Eggs from *M. sexta* (Lepidoptera, *Sphingidae*) were obtained from the Max Planck Institute for Chemical Ecology (Jena, Germany).

The eggs and larvae were maintained in a plexiglass cage under 27 °C, 16 h light/8 h dark, and 50% relative humidity, and fed with artificial diet [94].

4.2. Plant Growth Conditions and Fungal Inoculation

Tomato seeds were surface-sterilized in 10% (*v*/*v*) sodium hypochlorite (NaOCl, 12% ChemSolute, Th. Geyer, Berlin, Germany) for 4 min, then thoroughly rinsed in water. After sterilization, the seeds were placed on fine-grained moist vermiculite and germinated in the dark for 3 days at 28 °C, followed by 7 days in a plant growth chamber at 25 ± 3 °C, 16 h/8 h day: night cycle, 65–70% RH conditions. Ten days after germination, the seedlings were transplanted into 400 mL pots containing a sterile sand/vermiculite mixture (1:1, *v*/*v*). Inoculation with *R. irregularis* was performed by mixing the *R. irregularis* inoculum with the sand/vermiculite mixture at 10% (*v*/*v*) before transplanting [95]. Inoculation with *T. harzianum* was achieved by mixing the *T. harzianum* inoculum with the substrate to achieve a final density of 1×10^6 conidia g^{-1} before transplanting [95]. The plants were then placed in a completely randomized design in controlled climate chambers at 16 h/8 h day/night cycle, 65–70% RH. The plants were watered with tap water every second day, and once per week with Hoagland nutrient solution 50% water diluted [96]. Four weeks after transplanting, plants were used for the experiments. We did not find differences in the shoot biomass of the different treatments, and all the plants appeared similar, with no evident differences between the treatments.

4.3. Rhizophagus irregularis and Trichoderma harzianum Root Colonization

We confirmed that *R. irregularis* had efficiently colonized tomato roots in all the performed bioassays by incubating washed roots in 10% KOH (≥85% p.a., ROTH, Karlsruhe, Germany) and subsequently staining the fungal structures with 0.05% trypan blue (ROTH) [97]. The percentage of total root colonization was determined by the gridline intersection method [98] using a binocular stereo microscope (Leica DM 4000 B LED). The extent of mycorrhizal colonization was about 30–40% (percentage of total root length colonized by *R. irregularis*) in all the performed bioassays. We confirmed that *T. harzianum* had efficiently colonized the potting substrate in all the performed bioassays by using the plate count technique and PDA amended with 50 mg L^{-1} rose bengal (Applichem, Darmstadt, Germany) and 100 mg L^{-1} streptomycin sulphate (ROTH) [93]. Plates were incubated at 28 °C in darkness, and colony-forming unite (CFUs) were counted after 5 days. We found that the number of *T. harzianum* CFU in the potting media was similar to initial inoculation values in all the performed bioassays.

4.4. Bioassay for the Assessment of the Impact of Rhizophagus irregularis and Trichoderma harzianum on Manduca sexta Pupation and Adult Emergence

We placed a total of 25 *M. sexta* neonates on 5 plants (5 larvae per plant) of every treatment (inoculated with *R. irregularis* or *T. harzianum*, or non-inoculated). The larvae were placed on the third and fourth fully expanded leaf (counted from above) of every plant and allowed to feed freely on the entire plant. The plants were covered with fine mesh netting to prevent the escape of the larvae. After 7 days, the larvae were redistributed over different plants within the same treatment group (2 larvae per plant). During the bioassay, plants of the same treatment were exchanged for the damaged plants as needed to allow the larvae to feed ad libitum throughout the experiment. After 3 weeks, the surviving larvae were weighed and placed into plastic pupation boxes filled with vermiculite, which were placed in a growth chamber at 27 ± 3 °C, 16 h/8 h day/night cycle, and 50% RH. There, they were kept until they had reached the pre-pupal stage (approximately 3 days later). In the pupation boxes, the larvae were fed with artificial diet. The time required to reach the pre-pupal stage and the time required to complete the pupation process were recorded. After the pupation process had been completed, we visually checked the morphology of the developed pupae and recorded the number of pupae presenting morphological anomalies. From the pupae presenting a normal morphology, we collected data on pupal weight and sex determination. After the eclosion of the adults, we visually checked the morphology of

the moths and recorded the number of moths presenting morphological anomalies. The number of pupae that failed to eclose was also recorded.

4.5. Bioassay for the Assessment of the Impact of Rhizophagus irregularis and Trichoderma harzianum on Tomato Leaf Metabolome

One first-instar *M. sexta* larva was placed on the apical leaflet of the third fully expanded leaf (counted from above) using a clip cage, which was moved to a new leaf every two days. The plants had been inoculated with *R. irregularis* or *T. harzianum*, or were non-inoculated, as described above. Dead *M. sexta* larvae were immediately replaced by larvae of the same age that had been feeding on plants from the same treatment. Empty clip cages were placed on leaves of non-infested plants. A total of six biological replicates (plants) were used per treatment and time point. Larvae were replaced weekly by new first-instar larvae to avoid consumption of the entire plant. Three weeks after first challenging the plants, the leaf the larvae were actually feeding on was collected, flash-frozen in liquid nitrogen, and stored at −80 °C for metabolomics analyses.

4.6. Bioassay to Assess the Impact of Rhizophagus irregularis and Trichoderma harzianum on the Metabolome of Manduca sexta Gut and Fat Body

We used 45 tomato plants per microbial treatment (inoculated with *R. irregularis* or *T. harzianum* or non-inoculated). On each plant, we placed 4 second-instar larvae on the third fully expanded leaf (counted from above) using a clip cage, which was moved to a new leaf every two days. Within the first week, dead larvae were removed and replaced by new ones of the same age. After the first week of herbivory, since the caterpillars had grown too large for the clip cage, we replaced the cage by a net bag wrapped around the plant. During the bioassay, plants of the same treatment were exchanged for the damaged plants as needed to allow the larvae to feed ad libitum throughout the experiment. Three weeks after the start of the experiment, the larvae were collected and kept on ice while transferred to be stored at −20 °C for dissection.

4.7. Leaf Metabolomics

4.7.1. Extraction of Leaf Metabolites

We extracted leaf metabolites from 100 mg (fresh weight) of finely ground leaf material according to Rogachev and Aharoni [99,100], with the following modifications. For the sample extraction solution, high-performance liquid chromatography (HPLC grade, VWR International GmbH, Dresden, Germany) methanol and acetate buffer were used. For the preparation of the acetate buffer, 2.3 mL acetic acid (100% p.a., ROTH) and 3.41 g ammonium acetate (≥97% p.a., ACS, ROTH) were dissolved in 1L Millipore water. The pH of the buffer solution was adjusted to 4.8. For the extraction buffer, the acetate buffer (25%) was mixed with methanol (MeOH) (75%). For the extraction procedure, 100 ± 5 mg of each ground sample was weighed in a 2 mL Eppendorf tube. One milliliter of the extraction solution was added to each sample; the tube was tightly sealed and shaken for 10 s. Subsequently, each sample was sonicated for 5 min at 30 Hz using an FB 15061 sonicator (Elma, Singen, Germany) and then centrifuged for 15 min at 15,000× *g*. The supernatant was pipetted with a 1 mL pipette into a new-labeled Eppendorf tube, and the first Eppendorf tube was kept. By using the pipette, we added 1 mL of the extraction solution again to the pellet of the first extraction tube. The sealed tube was shaken again for 10 s and subsequently shaken for 5 min at 30 Hz in a sonicator. Then, it was centrifuged again for 15 min at 15,000× *g*. The two supernatants were combined, and the sample containing both supernatants was centrifuged for 10 min at 15,000× *g*; afterwards, 200 µL of the sample was transferred in an HPLC vial, and 800 µL of the extraction solution was added, resulting in a 1:5 dilution of the samples. The samples were then transferred to liquid chromatography–quadrupole time of flight–mass spectrometry for analysis.

4.7.2. Liquid Chromatography–Quadrupole Time of Flight–Mass Spectrometry and Data Analysis of the Leaf Extracts

We obtained mass spectra of separated metabolites from the leaf samples by using UPLC–MS. The liquid chromatograph (Dionex UltiMate™ 3000, Thermo Scientific, Sunnyvale, CA, USA) was equipped with a C18 analytical column (Acclaim TM RSLC 120; 2.1 × 150 mm, 2.2 µm particle size, 120 Å pore size). The column was kept at 40 °C. The mobile phase was composed of solvent A, water/formic acid (99–100%, VWR) (0.05% v/v), solvent B, and acetonitrile (HPLC grade, VWR)/formic acid (0.05% v/v) at the flow rate of 400 µL min^{-1}. The multi-step gradient for solvent B was 0–1 min, 5 %; 1–4 min, 28%; 4–10 min, 36%; 10–12 min, 95%; 12–14 min, 95%; 14–16 min, 5%; 16–18 min, 5%. The chromatograph was coupled to a maXis impact HD MS-qToF (Bruker Daltonics) operated in positive polarity. ESI source conditions were end plate offset = 500 V, capillary = 4500 V, nebulizer = 2.5 bar, dry gas = 11 L min^{-1}, dry temperature = 220 °C. Transfer line conditions were funnels 1 and 2 = RF 300 Vpp, isCD energy = 0 eV, hexapole = 60 Vpp, quadrupole ion energy = 5 eV, low mass = m/z 50, collision cell energy = 10 eV, collision RF = 500 Vpp, transfer time = 60 µs, pre-pulse storage = 5 µs. The mass spectrometer operated with a mass range of m/z 50–1500 and a spectral acquisition rate of 3 Hz. Sodium formate clusters (10 mM) were used for calibrating the m/z values. The clusters mix consisted of 250 mL isopropanol (≥99% for LC–MS, VWR), 1 mL formic acid, and 5 mL 1M NaOH (≥99% p.a., ISO, ROTH), and the final volume was adjusted to 500 mL. For the identification of the steroidal glycoalkaloid α-tomatine in the leaf extracts, we used the commercial standard α-tomatine (500 mg, 0602, Lot: 12021719, Extrasynthase, Genay, France).

For the data analysis, Bruker *.d* files were transformed to *.abf* files using the program Abf Converter (https://www.reifycs.com/AbfConverter/, Tokyo, Japan, accessed on 5 May 2021). The *.abf* files were processed with the program MS-DIAL (v. 4.00, available online: http://prime.psc.riken.jp/compms/msdial/main.html, RIKEN, accessed on 5 May 2021) with the following parameters: data collection: mass accuracy: MS1 tolerance = 0.01 Da, retention time begin = 0.7 min, retention time end = 10 min, mass range begin = m/z 50, mass range end = m/z 1500; peak detection parameters: minimum peak height = 1000 amplitude, mass slice width = 0.1 Da, smoothing method = linear weighted moving average, smoothing level = 3 scans, minimum peak width = 5 scans; alignment parameter settings: retention time tolerance = 0.2 min, MS1 tolerance = 0.015 Da. We normalized the alignments against the total ion chromatogram. We exported the normalized data matrix containing all the alignments as a *.txt* file (spectra type = centroid). The level of confidence for the annotation/identification of the metabolomic features described in our analyses was determined on the basis of Malinowska and Viant [45].

4.8. Dissection of Manduca sexta Larvae and Separation of the Gut and Fat Body Samples

Before the dissection, the larvae were kept cool on ice for 30 min to sedate them. By using a sterile scalpel, each larva was dissected on two points. At first, the red horn at the bottom of the larval body was removed. Then, each larva was cut open at the ventral side from the head down to the posterior end using surgical scissors. For the separation of the samples, the central gut and the fat body tissues were carefully removed from the larval body using tweezers and subsequently separated one from each other. Guts and fat body samples from individual larvae were stored separately in a 1.5 mL safety lock Eppendorf tube, and the cap of each tube was punctured by using a sterile syringe before use. The samples were stored at −80 °C until further processing. Afterwards, the samples were freeze-dried and stored again at −80 °C until metabolite extraction.

4.9. Insect Metabolomics

4.9.1. Extraction of Metabolites from the Gut and Fat Body of *Manduca sexta*

The gut and fat body sample dry masses were assessed before extraction. To handle the variability in the samples weight, we extracted the metabolites by following the ratio w/v = 1:2, meaning that in each sample tube, the volume of the extracting solution added in

milliliters would be double that of the sample's mass in milligrams. Using this procedure, we added the appropriate volume of 70% MeOH (≥99.9%, HiPerSolv CHROMANORM® til HPLC, VWR, Søborg, Denmark), with 10 ppm palmitic acid methyl ester (Merck, Søborg, Denmark) and 10 ppm sorbitol (99%, Merck, Søborg) as internal standards, to each sample. The samples were vigorously vortexed for 30 s at 3000 rpm and then centrifuged at 12,000× *g* for 20 min at 4 °C. For LC–MS analyses, 50 µL of the supernatant (extracts) was transferred to a 200 µL glass insert in a 2 mL labelled glass vial. The vials (32 × 11.6 mm, Mikrolab Aarhus A/S, Denmark) containing the extracts were sealed with cap with septum and placed in the fridge (−20 °C) until analysis on LC–MS.

4.9.2. Ultra High Performance Liquid Chromatography–Quadrupole Time of Flight–Mass Spectrometry and Data Analysis of the Insect Extracts

UHPLC–MS analyses were performed with the extracts of the gut and fat body of *M. sexta* larvae by using a Dionex Ultimate 3000RS UHPLC system (Thermo Fisher Scientific) system coupled to a Compact™ (QqToF) mass spectrometer (Bruker Daltonics) with an electrospray ionization source. The UHPLC system was equipped with a DAD detector (acquiring from 190–800 nm), temperature-controlled auto-sampler (10 °C), and column oven (set to 40 °C).

Samples were analyzed on a Kinetex XB-C18 UHPLC column (100 × 2.1 mm, 1.7 µm, 100 Å pore size; Phenomenex) and eluted with a flow rate of 0.3 mL/min. The mobile phase composed of a gradient of solvent A (0.05 % formic acid (LC–MS, Ultra UHPLC–MS, Fluka, Rosklid, Denmark) in water) and solvent B (0.05% formic acid in acetonitrile (≥99.9%, HiPerSolv CHROMANORM® til HPLC, suitable for UPLC/UHPLC, VWR, Søborg, Denmark)). The initial composition was 98% A and 2% B, which was held for 1 min before the composition of solvent B was increased linearly to 100% over 19 min. The column was washed with 100% solvent B for 7 min, before rapidly returning to 2% B over 1 min, and subsequently re-equilibrated at 2% B for 7 min prior to the next injection. The total run time was 35 min.

The qToF mass spectrometer was operated in full scan positive ion mode with the following instrument settings: m/z 50–1200: nebulizer gas (nitrogen), 2.0 bar; drying gas (nitrogen), 8 L/min; drying gas temperature, 220 °C; capillary voltage, 4000 V; full scan MS spectra acquisition rate, 2 Hz; set ion energy, 4.0 eV; set isolation mass, m/z 100.00; collision energy, 7.0 eV; and set collision cell RF, 500.0 Vpp. For the MS/MS spectra, the qToF mass spectrometer was operated as described previously with the following conditions for the collision cell (quadrupole): set ion energy, 4.0 eV; set isolation mass, m/z 100.00; collision energy, 7.0 eV; set collision cell RF, 500.0 Vpp. Every chromatogram was calibrated to provide accurate mass by automated infusion of sodium formate clusters (10 mM) at the beginning of each run. For the identification of the steroidal glycoalkaloid α-tomatine in the insect extracts, the commercial standard α-tomatine (CAS Number: 17406-45-0, Merck, Germany) was used. All data acquisition was automated using a combination of Chromeleon Express (Thermo Fisher Scientific), Compass qTOF Control (Version 4.0.15.3248, Bruker Daltonics), and Hystar (Version 3.2 SR4, Bruker Daltonics) software. For data analysis, DataAnalysis software (Version 4.3, Bruker Daltonics) was used. Metabolomic analyses were firstly performed using the Bruker MetaboScape Mass Spectrometry Software, version 5.0 (Build 683) (Bruker Daltonic GmbH, Bremen, Germany). The polarity mode was positive, and the workflow followed was T-ReX 3D (LC-QToF). The parameters set for the analyses were the following: minimum number of features for extraction = 4, presence of features in minimum number of analyses = 3, noise threshold = 2000, minimum peak length = 10, recursive feature extraction = 8, range for the retention time windows from 0.25 to 20 min, mass range from m/z 50 to 3000, ion deconvolution = $[M+H]^+$, and EIC = 0.8. The chemical formula of the metabolites was automatically annotated by using the software's Smart Formula option, and subsequently, each metabolite was annotated by using the spectral libraries and the MS/MS libraries available. The MS/MS libraries used have been created in international institutes on the basis of commercial standards.

4.10. Statistical Analysis

Pearson's chi-squared test was used to determine whether there were statistically significant differences between the observed and expected frequencies of female and male *M. sexta* pupae that developed after feeding on the tomato plants of the three different treatment groups. One-way analysis of variance (ANOVA) was performed to evaluate the effect of the different treatments on the days required until pre-pupation, the days required for pupation, and the weight of the pupae showing normal morphology. For the statistical analysis of the tomato leaf data set, we used the web-based metabolomic data processing tool MetaboAnalyst (5.0) (available online: https://www.metaboanalyst.ca/, accessed on 5 May 2021) [101]. The data were filtered by taking the interquartile range and then pareto scaled. One-way analysis of variance (ANOVA) followed by Fisher's least significant difference method (Fisher's LSD) was performed in order to explain the variation within the data set. In addition, principal component analysis (PCA) was performed to identify significant differences among the treatments. For the statistical analyses of the *M. sexta* gut and fat body data set, firstly, the Bruker MetaboScape Mass Spectrometry Software (version 5.0) was used. A *t*-test was performed on the basis of the results of the processed spectral data. The resulting intensity table was exported and used for further statistical analysis using the web-based metabolomic data processing tool MetaboAnalyst (5.0) (available online: https://www.metaboanalyst.ca/, accessed on 5 May 2021) [101]. The data were filtered by interquartile range and transformed by using the generalized log transformation (glog 2). Pair-wise comparisons were then conducted between the non-inoculated and beneficial microbe-inoculated gut and fat body samples separately. These pair-wise comparisons included non-parametric *t*-tests with the level of significance set at $\alpha = 0.05$ and volcano plots with a fold change threshold (*x*-axis) equal to 2 and *t*-test threshold (*y*-axis) equal to 0.05. The level of confidence for the annotation/identification of the metabolomic features described in all our analyses was determined on the basis of the work of Malinowska and Viant [45].

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/metabo11110731/s1, Figure S1: qToF intensity of the metabolomic feature annotated as (**A**) α-tomatine; (**B**) an isomer of α-tomatine; (**C**) an isomer of α-tomatine; (**D**) α-dehydrotomatine; (**E**); an isomer of α-dehydrotomatine; (**F**) an isomer of α-dehydrotomatine. Samples correspond to leaves of tomato plants that were non-inoculated or root-inoculated with Rhizophagus irregularis and not challenged or shoot-challenged with Manduca sexta herbivory for three weeks. The qToF intensity was pareto scaled. The box plots summarize the scaled values of the qToF intensity in each group, the whiskers represent the values outside the middle 50%, the bisecting lines represent the median of the values, the yellow diamond indicates the mean qToF intensity in each group and the dots represent the samples in each group. Asterisks indicate statistically, Figure S2. qToF intensity of the metabolomic feature annotated as (**A**) α-tomatine; (**B**) an isomer of α-tomatine; (**C**) an isomer of α-tomatine; (**D**) an isomer of α-dehydrotomatine; (**E**) an isomer of α-dehydrotomatine. Samples correspond to leaves of tomato plants that were non-inoculated or root-inoculated with Trichoderma harzianum and not challenged or shoot-challenged with Manduca sexta herbivory for three weeks. The qToF intensity was pareto scaled. The box plots summarize the scaled values of the qToF intensity in each group, the whiskers represent the values outside the middle 50%, the bisecting lines represent the median of the values, the yellow diamond indicates the mean qToF intensity in each group and the dots represent the samples in each group. Asterisks indicate statistically significant differences among the groups according to Fisher's Least Significance Difference (LSD) test ($p \leq 0.05$)., Table S1. Results for the one-way ANOVA for the effect of root colonization by Rhizophagus irregularis on the qToF intensity of steroidal glycoalkaloids in the leaf metabolome of tomato plants after Manduca sexta herbivory for three weeks. Each metabolomic feature is given by its mass to charge ratio (m/z) and its retention time (rt). FDR: means False Discovery Rate., Figure S3. qToF intensity of the metabolomic feature annotated as α-tomatine in the gut (**A**) and fat body (**B**) samples of Manduca sexta larvae reared for three weeks on leaves of plants that were non-inoculated and root-inoculated by Rhizophagus irregularis. The qToF intensity was log transformed. The box plots summarize the values of the qToF intensity in each group, the whiskers represent the values outside the middle 50%, the bisecting

lines represent the median of the values, the yellow diamond indicates the mean qToF intensity in each group and the dots represent the samples in each group. Asterisks indicate statistically significant difference between the treatments according to Student *t*-tests ($p \leq 0.05$)., Figure S4. qToF intensity of the metabolomic feature annotated as α-tomatine in the gut (**A**) and fat body (**B**) samples of Manduca sexta larvae reared for three weeks on leaves of plants that were non-inoculated and root-inoculated by Trichoderma harzianum. The qToF intensity was log transformed. The box plots summarize the values of the qToF intensity in each group, the whiskers represent the values outside the middle 50%, the bisecting lines represent the median of the values, the yellow diamond indicates the mean qToF intensity in each group and the dots represent the samples in each group. Asterisks indicate statistically significant difference between the treatments according to Student *t*-tests ($p \leq 0.05$). Table S2. Results for the one-way ANOVA for the effect of root colonization by Trichoderma harzianum on the qToF intensity of steroidal glycoalkaloids in the leaf metabolome of tomato plants after Manduca sexta herbivory for three weeks. Each metabolomic feature is given by its mass to charge ratio (m/z) and its retention time (rt). FDR: means False Discovery Rate.

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Manuscript 2

FORM 1

Manuscript No. 2

Manuscript title: "Root colonization by the mutualistic fungi *Trichoderma harzianum* or *Rhizophagus irregularis* alters the transcriptomic and metabolomic defense response of tomato plants to *Manduca sexta* L. herbivory"

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Author	Conceptual	Data analysis	Experimental	Writing the manuscript	Provision of material
Colina Francisco		50%			
Papantoniou Dimitra	20%	25%	20%	80%	
Vergara Fredd		25%			
Rivero Javier	30%		60%		50%
van Dam Nicole	20%				
Martínez-Medina Ainhoa	30%		20%	20%	50%
Total:	100%	100%	100%	100%	100%

Root colonization by the mutualistic fungi *Trichoderma harzianum* or *Rhizophagus irregularis* alters the transcriptomic and metabolomic defense response of tomato plants to *Manduca sexta* L. herbivory

1. Introduction

Insect herbivory is one of the most important factors causing severe economic losses in agricultural production. It is estimated that approximately 10-30% of crop production is lost due to insect pests on a global scale (Savary et al., 2019). The continuous "battle" between insects and plants has led the latter to the development of complex, sophisticated defense mechanisms to confront their enemies (Karban & Baldwin, 2007; Walling, 2000; War et al., 2012). Direct plant defense mechanisms include the formation of physical barriers against insect herbivores and/or the synthesis of compounds that exert repellent, anti-nutritive or toxic effects on the herbivore enemies (Fürstenberg-Hägg et al., 2013).

Early events in plant-herbivore interactions include the recognition of the attacker, and the activation of signal transduction pathways throughout the attacked plant's tissues (Mithofer & Boland, 2008). Detection of leaf damage due to herbivory results in the depolarization of the plasma membrane potential, followed by protein phosphorylation, and activation of plasma membrane proteins (Arimura et al., 2009; Maffei et al., 2004; Maffei et al., 2007a; Mithöfer et al., 2009). These processes lead to the mobilization and generation of diverse signaling molecules, such as free cytocolic calcium (Ca^{2+}), nitric oxide, and reactive oxygen species (ROS) (Maffei et al., 2007b). Subsequently, a wide signaling network mediated by transcription factors and phytohormones is activated in the attacked plants (Schuman & Baldwin, 2016; Wu & Baldwin, 2010).

Phytohormone-mediated signal transduction pathways regulate the induced defense responses to herbivory. Among phytohormone pathways, the pathway regulated by jasmonic acid (JA) has a prominent role in the regulation of plant defenses against chewing herbivores, such as beetles and caterpillars (Howe & Jander, 2008; Wasternack & Hause, 2013). Other hormones such as salicylic acid (SA), ethylene (ET) and abscisic acid (ABA) interact with the JA pathway in the orchestration of defenses (Heidel-Fischer et al., 2014). Several studies indicate that the cellular responses to phytohormone signals are highly interconnected. Indeed, both positive and negative cross-talk occur among hormonal-regulated pathways, allowing the plant to fine-tune the appropriate defense responses (Bonaventure, 2014; Bostock et al., 2001; Glazebrook et al., 2003; Spoel et al., 2003; Van Wees et al., 2000).

In their complex environments, plants also interact with mutualistic organisms such as root mutualistic microbes (Pineda et al., 2010). Root mutualistic microbes can enhance plant growth and protect plants against biotic and abiotic stresses (Lee Díaz et al., 2021). Root colonization by certain mutualistic microbes induces an immune response in plants, rendering the entire plants

more resistant to the attack of pathogens and pests. This phenomenon is known as Induced Systemic Resistance (ISR), and it is characterized by a priming effect that results in a more efficient activation of the plant immune responses upon attack. ISR provides plants with broad-spectrum resistance against a variety of pathogens and pests that attack their shoots and roots (Pozo & Azcón-Aguilar, 2007; Sanchez et al., 2005; Van Wees et al., 2008; Zamioudis & Pieterse, 2012).

Among plant mutualistic microbes, two well-studied examples are the arbuscular mycorrhizal fungi (AMF) and *Trichoderma* fungi. AMF are obligate root biotrophs, which form symbiotic associations with approximately 80% of terrestrial plants (Brundrett & Tedersoo, 2018). Mycorrhizal plants exhibit increased water and nutrient uptake (in particular phosphorus, nitrogen and micronutrients), and enhanced resistance to abiotic and biotic stress factors (Hodge & Storer, 2015; Smith & Read, 2010; Sally E Smith & F Andrew Smith, 2011). In return, AMF obtain photosynthesis-derived carbohydrates from the plants (Bago et al., 2003; Lanfranco et al., 2018; Solaimanand & Saito, 1997). The filamentous fungi of the genus *Trichoderma* spp. are commonly found in the rhizosphere and have been broadly studied due to their mycoparasitic properties and their ability to produce a plethora of bioactive compounds (Harman et al., 2004; Velázquez-Robledo et al., 2011; F Vinale et al., 2008; Francesco Vinale et al., 2008). Moreover, selected *Trichoderma* isolates can stimulate plant growth, enhance nutrient uptake, and elicit plant defenses against abiotic and biotic stress factors (Lorito & Woo, 2015; Shoresh et al., 2010; Studholme et al., 2013). Notably, colonization of roots by arbuscular mycorrhiza and *Trichoderma* fungi can lead to systemic biochemical changes in the composition and concentration of primary and secondary metabolites in above-ground plant tissues (Andrade et al., 2013; Coppola, Diretto, et al., 2019; Fiorentino et al., 2018; Mayo-Prieto et al., 2019; Rivero et al., 2021; Schweiger et al., 2014; Schweiger & Müller, 2015; Sofo et al., 2012; Song et al., 2011; Zhou et al., 2018). Such changes can affect the quality of host plants for insect herbivores, affecting thus herbivore development, reproduction, and survival.

There has been an intense research effort to unravel the main mechanisms regulating the impact of beneficial microbes on plant immunity during the last years. However, our knowledge on the mechanistic basis of ISR is still highly fragmented, based on targeted analysis of few traits, and limited to one level of plant biological organization (Gruden et al., 2020). The current implementation of systems biology approaches is contributing to significantly overcome such biases, and to better understand the broad spectrum of mechanisms regulating plant-microbe-insect interactions (Coppola, Diretto, et al., 2019; Gupta et al., 2022; Kaling et al., 2018; Mashabela et al., 2022). Here, we pursued a integrative -omics approach to address how the two different root mutualistic fungi, the arbuscular mycorrhizal fungus *Rhizophagus irregularis* and the plant growth-promoting fungus *Trichoderma harzianum,* affect the transcriptomic and metabolomic response of tomato plants to Manduca *sexta* herbivory. With this aim, we used a high-throughput RNA sequencing and metabolomics analysis, followed by the integration of both datasets. The untargeted analysis, involving the independence of previous assumptions has allowed us to hypothesize plant-related mechanisms involved in microbe-mediated induced resistance by root mutualistic fungi against insect herbivores.

2. Material and methods

2.1 Plant, fungal and insect material

Seeds of *Solanum lycopersicum* cv. Moneymaker (Intratuin, the Netherlands) were surface-sterilized in 4% (v/v) sodium hypochlorite (NaOCl) for 10 minutes, then thoroughly rinsed in water. After sterilization, the seeds were placed on finely coarsed, moist vermiculite and kept in the dark for three days at 28°C. Inoculum of the arbuscular mycorrhizal fungus *Rhizophagus irregularis* (INOQ-Sprint) was purchased from INOQ GmbH (Schnega, Germany) (https://inoq.de/), with 220 mycorrhizal units per mL in sand. *Trichoderma harzianum* T-78 (CECT 20714, Spanish Type Culture Collection; provided by J.A. Pascual, CEBAS-CSIC) was cultured on potato dextrose agar plates for 5 days at 28°C in dark, as described by Martínez-Medina et al. (2014). T-78 inoculum was prepared on commercial oat, bentonite and vermiculite according to Martínez-Medina et al. (2009). *Manduca sexta* (Lepidoptera, *Sphingidae*) eggs were obtained from the Max Planck Institute for Chemical Ecology (Jena, Germany). The *M. sexta* culture was reared on artificial diet and maintained according to Grosse-Wilde et al. (2011).

2.2 Plant growth conditions

Ten days after germination, the seedlings were transplanted into 350 mL pots filled with a sand-vermiculite mixture (1:1, v/v). Microbial inoculation was achieved by mixing the microbial inocula through the sand-vermiculite mixture before transplanting the seedlings. For the mycorrhizal treatment, the sand-vermiculite mixture was inoculated with 10% v/v of *R. irregularis* inoculum, according to Papantoniou et al. (2021). Inoculation with *Trichoderma harzianum* was achieved by mixing the *T. harzianum* inoculum in the sand-vermiculite mixture to a final density of 1×10^6 conidia g^{-1} before transplanting, according to Papantoniou et al. (2021).

The plants were grown under greenhouse conditions (T= 25-27°C, RH= 65-70%). They were watered with tap water every second day, and half-strength Hoagland solution (Hoagland & Arnon, 1950) was provided once per week to support them with the essential nutrients.

2.3 Fungal colonization

Five weeks after transplanting, root colonization by *R. irregularis* was assessed after clearing washed roots in 10% KOH and subsequent staining of fungal structures with 5% ink (Koh-I-Noor) and 2% acetic acid (Vierheilig et al., 2005). To calculate the percentage of total root colonization, we used the gridline intersection method (Giovannetti & Mosse, 1980) using a binocular stereo microscope. We found that the percentage of root colonization was about 30-40% in all

mycorrhiza-inoculated plants. To quantify the amount of colony forming units (CFU) of *T. harzianum*, we sampled substrate from the rhizosphere. We used the plate count technique using potato dextrose agar (PDA), amended with 50 mg L^{-1} rose bengal and 100 mg L^{-1} streptomycin. Plates were incubated at 28°C in darkness, and CFUs were counted after 5 days. The CFUs were calculated per gram of dry (1 week, 50°C) soil (Martínez-Medina et al., 2011). We found that the populations of T-78 in the soil were similar to the inoculation level (1×10^6 conidia g^{-1}) in all T-78 inoculated plants.

2.4 *Manduca sexta* infestation

Five weeks after transplanting, the corresponding plants were infested with two neonate *Manduca sexta* larvae. Larvae were placed on the three apical leaflets of the third true leave (counted from the soil), inside a clip cage (30 mm diameter). Empty clip cages were used similarly for non-herbivory treatments. The experiment consisted of 6 treatments: non-microbe plants without herbivory (hereafter referred as Nm), non-microbe plants challenged with *M. sexta* (hereafter referred as Nm_h), *R. irregularis* plants without herbivory (hereafter referred as Rhi), *R. irregularis* plants challenged with *M. sexta* (hereafter referred as Rhi_h), *T. harzianum* plants without herbivory (hereafter referred as Tri) and *T. harzianum* plants challenged with *M. sexta* (hereafter referred as Tri_h). Each treatment consisted of 6 independent plants as biological replicates. After 24 h, the larvae were removed, and the leaflets contained within the clip cage were harvested and immediately flash frozen in liquid nitrogen and stored at −80°C.

2.5 RNA isolation and sequencing

For RNA-seq analysis, three biological replicates per treatment were used, each consisting of pooled material from two plants. We extracted total RNA from tomato leaf samples using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. All samples were treated with DNase I on column using the Qiagen RNase-Free DNase Set. RNA quality was evaluated by determining the RNA Integrity Number (RIN) using Agilent 2100 Bio-analyzer and RNA LabChip in the Agilent 2100 Bio-Analyzer (Agilent Technologies, Santa Clara, CA, United States). For the library preparation, the samples with a RIN value ≥9 were used. The strand specific paired-end libraries were constructed using the Illumina TruSeq Stranded mRNA LT Sample Prep Kit (Illumina) and sequenced on the NovaSeq 6000 platform (Illumina) from both ends.

2.6 Read mapping and transcript quantification

The quality of raw RNA-seq reads was evaluated using FastQC (Andrews, 2010) before a trimming step using Trimmomatic v0.39 (Bolger et al., 2014) for the removal of adapter sequences and low-quality reads. Briefly, low-quality sequence stretches at the end of the reads were identified and trimmed through a sliding window approach. This "sliding" approach averaged the quality of a stretch of 4-mers from the end of the reads and trimmed it whenever their average base calling quality dropped below 15 (Phred+33 encoding). After trimming, the algorithm "slided" evaluated the quality of the next 4-mer and continued trimming until the identification of a block with an average base calling quality above 15 (SLIDINGWINDOW:4:15). For the removal of adapter sequences, Illumina adapter sequences were fed to the Trimmomatic algorithm allowing a maximum of two mismatches for a positive identification of an adapter sequence under both available alignment modes, normal and palindrome. Alignment quality thresholds of 30 and 10 were set for the palindrome and normal alignment modes, respectively, and a minimum of 4-mers were required for identifying and cutting an adapter sequence in palindrome mode. Both reads were kept for all read-throughs (ILLUMINACLIP: adapter_sequences.fa:2:30:10:4:TRUE). After the trimming steps, all reads below 50 bp were discarded (MINLEN:50). Ribosomic RNA reads were filtered out of the trimmed reads using SortMeRNA v4.2 (Kopylova et al., 2012). High quality trimmed and filtered reads were pseudomapped to a reference sequence including the tomato ITAG4.1 transcriptome (Hosmani et al., 2019) and transcript abundances were estimated afterwards using the suite Salmon v1.4 (Patro et al., 2017). Briefly, by using the Salmon index we built an index of the reference sequence including a concatenation of all ITAG4.1 transcript sequences plus the sequence of the tomato genome assembly SL4.0 as decoy using k-mers of length 31. Then, we ran Salmon quant over the previously generated index and different subsets of filtered and trimmed samples including all samples (herbivory samples and non-herbivory). Different attributes were specified to Salmon quant for correcting biases observed during the quality control steps (seqBias, gcBias, posBias), and enhancing the accuracy of the quantification estimates (validateMappings, useVBOpt).

We employed the DESeq2 package (Love et al., 2014) for the identification of differentially expressed genes (DEGs) by using the Wald test function included in the package. Log2 fold changes (LFC) resulting from pairwise comparisons were corrected "shrinked" using the apeglm package (Zhu et al., 2019). Only the genes with an absolute shrink LFC value above 1 and a Wald test q-value below 0.01 were considered as differentially expressed. We also used DESeq2 for the pre-processing of the transcriptomic data for all the downstream multivariate and integrative analyses. Briefly, the different datasets were normalized by the median of ratios method and rlog transformed (Love et al., 2014). Likelihood Ratio Test (LRT) was used as an unsupervised filter for the rlog transformed datasets. Only variables with an LRT q-value below 0.05 were kept for downstream analysis including functional enrichment, clustering, and multivariate analyses as principal component analysis (PCA) and sparse Partial Least Squares (sPLS) analysis.

2.7 Metabolite extraction and metabolome analysis

For metabolomic analysis, six biological replicates per treatment were used, each one consisting in one independent plant. We extracted metabolites from tomato leaf samples and quantified those following previous protocols (De Vos et al., 2011; Moreno-Pedraza et al., 2019; Rogachev & Aharoni, 2011) with some modifications. Briefly, sample extract aliquots were diluted to 1:5 and 1:50 in extraction buffer [acetate buffer (25%) and methanol (75%)] –the 1:50 dilution allowed us to correctly detect the tomatine peak without exceeding the mass analyzer detection limit- 1 µL from each sample dilution (1:5 and 1:50) was injected in an Ultra Performance Liquid Chromatography (UPLC) (Dionex 3000, Thermo Scientific) equipped with a C18 column (Acclaim TM RSLC 120, CN: 071399) kept at 40 °C. The mobile phases (LC-MS grade solvents) were composed of solvent A: 0.05% (v/v) aqueous formic acid and solvent B: 0.05% (v/v) formic acid in acetonitrile. The multi-step gradient for solvent B was 0−1 min 5%, 1−4 min 28%, 4−10 min 36%, 10−12 min 95%, 12−14 min 95%, 14−16 min 5%, 16−18 min 5%. The flow was 400 µL min^{-1}. We detected compounds using a maXis impact HD Mass Spectrometer-quadrupole Time-of-Flight (MS-qToF) (Bruker Daltonics). Data were acquired in positive mode with settings similar to Moreno-Pedraza et al. (2019). Subsequently, we processed the data according to Moreno-Pedraza et al. (2019) with slight modifications for feature detection, retention time correction, and feature alignment. The parameters were; mass accuracy: MS1 tolerance = 0.01 Da, retention time-begin = 0.7 min, retention time-end = 10 min, mass range-begin = 50 mass to charge ratio (m/z), mass range-end = 1500 m/z; peak detection parameters: minimum peak height = 1000 amplitude, mass slice width = 0.1 Da, smoothing method = linear weighted moving average, smoothing level = 3 scans, minimum peak width = 5 scans; alignment parameters settings: retention time tolerance = 0.05 min, MS1 tolerance = 0.015 Da. Samples were normalized by the total intensity of the chromatogram. We generated three different datasets including all samples, herbivory samples and non-herbivory samples. Resulting datasets were z-score transformed prior to their use in PCA and sPLS analyses.

2.8 Functional enrichment, Principal Component Analysis (PCA) and sparse Partial Least Squares (sPLS) analyses

We performed GO functional enrichment analysis over ranked DEGs lists using g:Profiler (Raudvere et al., 2019). Briefly, DEGs were split between upregulated and downregulated, and then ranked by their shrank LFC values. Ranked lists were used as the ordered input for g:Profiler. In parallel, we also performed a hierarchical clustering analysis over the abundance of all transcripts included in PCA and sPLS analyses based on their MapMan functional categories. Hierarchical clustering was carried out using the pRocessomics package (https://github.com/Valledor/ pRocessomics).

Integration of transcriptome and metabolome levels was performed through an sPLS regression analysis using transcriptomic and metabolomic datasets as prediction and response matrices,

respectively. Samples in the metabolome data matrix belonging to the same pool in the transcriptome matrix were averaged to correct for asymmetries in the number of biological replicates between the involved -omics levels incompatible with the sPLS analysis. PCA and sPLS (Lê Cao et al., 2009) analyses were performed using the pRocessomics package. All sPLS-derived transcript-metabolite correlation networks were processed in Cytoscape (Cytoscape consortium 2021).

3. Results and discussion

3.1 Transcriptomic dataset description

The transcriptomics dataset described changes in the expression of 24,859 genes. Within this gene set, 7,352 genes were differentially expressed (LRT, α 0.05) (Supplementary table S2) and 2,059 showed expression changes in the transition from non-herbivory to herbivory conditions (|shrink LFC| > 1, α 0.01) (Figure 1A). Among the genes differentially expressed in the transition from non-herbivory to herbivory, we found a core of 706 common genes that were differentially expressed among the Nm, Rhi and Tri treatments. We also found genes that were differentially expressed exclusively in the case of Rhi (321), Tri (401) and Nm plants (176) (Figure 1A). Interestingly, 220 common genes were differentially expressed under herbivory in both Rhi and Tri plants. When focusing only on the data under herbivory, we found that only 6 genes were differentially expressed between Nm and Rhi plants; and 13 genes were differentially expressed between Nm and Tri plants (Figure 1B).

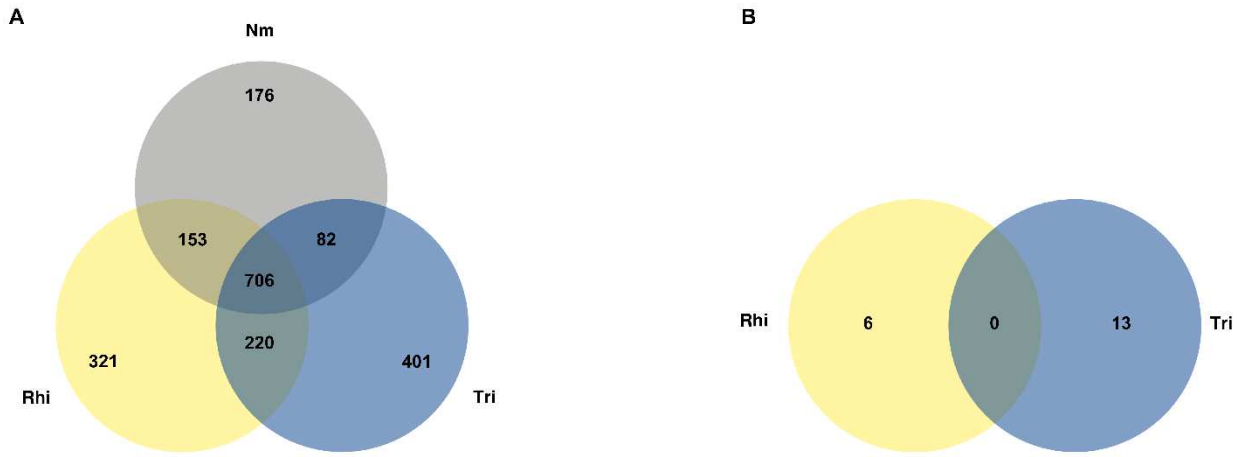

Figure 1. Venn diagram visualization of the differentially expressed genes (DEGs). (A) DEGs of tomato plants (non-herbivory vs. herbivory) in all three treatments: non-microbe (symbolized as Nm and depicted in grey), *Trichoderma harzianum*-inoculated (symbolized as Tri and depicted in blue) and *Rhizophagus irregularis*-inoculated (symbolized as Rhi and depicted in yellow). **(B)** DEGs

of tomato plants when comparing Nm vs. Rhi (left side, depicted in yellow), and Nm vs. Tri (right side, depicted in blue) after herbivory.

3.2 Results from GO enrichment and MapMan analyses of the transcriptomic dataset

The gene ontology (GO) enrichment analysis over non-herbivory vs. herbivory LFC ranked DEGs showed herbivory response elements commonly shared among non-microbe and microbe-inoculated plants (Figure 2A). Among the herbivory-triggered upregulated DEGs, we found genes coding for peptidase inhibitor activity, transferase activity, catalytic activity, transferring of acyl groups and lipid metabolic process. Interestingly, the transferase/catalytic activity categories were further enriched in the microbe-inoculated (Rhi and Tri) plants. Protease inhibitors are substantial compounds of direct plant defense responses, acting as effective anti-nutritional defense against insect herbivores (Howe & Jander, 2008). Similar to our results, several studies have demonstrated that mycorrhizal and *Trichoderma* fungi can enhance the expression of different protease inhibitors upon herbivory leading to enhanced resistance (Alınç et al., 2021; Bacht et al., 2019; Coppola, Cascone, et al., 2019; Coppola, Diretto, et al., 2019; Kaling et al., 2018; Li et al., 2019; Song et al., 2013).

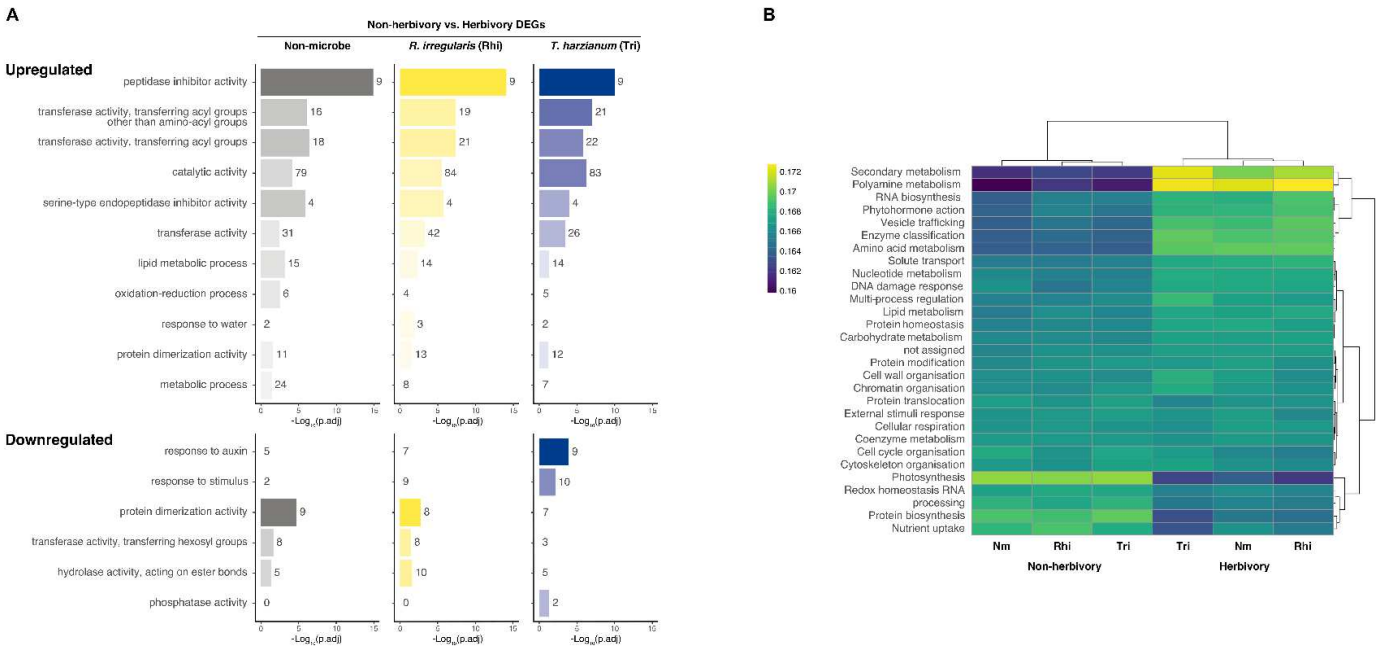

Figure 2. Visualization of Gene Ontology (GO) enrichment and MapMan analyses for the transcriptomic data set. (A) GO enrichment analysis (over non-herbivory vs. herbivory) for the differentially expressed genes set (DEGs) in all three treatments: non-microbe (symbolized as Nm, depicted in grey) *Trichoderma harzianum*-inoculated (symbolized as Tri, depicted in blue)

and *Rhizophagus irregularis*-inoculated (symbolized as Rhi, depicted in yellow). **(B)** MapMan-based hierarchical clustering (over non-herbivory vs. herbivory) for the DEGs in all three treatments (non-microbe-Nm, *Trichoderma harzianum*-inoculated-Tri and *Rhizophagus irregularis*-inoculated-Rhi). The intensity of differential expression among the hierarchically clustered genes is given in a color scale from yellow to dark blue.

In addition, we found microbe-specific changes among the upregulated categories, as for instance, the higher enrichment in the category "response to water" that was observed specifically for Rhi plants. This finding was further reinforced by the upregulation of three dehydrins in the same samples, including the dehydrin *TAS14*. Members of the group 2 of Late-Embryogenesis-Abundant (LEA) proteins, called dehydrins, are mainly found in plants and are typically accumulated in dehydrating plant tissues, in seeds and/or in tissues subjected to environmental stresses including drought, salinity and low temperature (Veeranagamallaiah et al., 2011). Upon herbivory, insect injuries can cause increases in water loss (Dunn & Frommelt, 1998; Ostlie & Pedigo, 1984; Sun et al., 2015). Therefore, the higher enrichment observed in the category "response to water", including the upregulation of the three dehydrins in Rhi plants, may be linked to the ability of AMF to ameliorate water deficit stress upon herbivory. Along the same lines, several studies have reported that AMF are capable of helping plants to resist water deficit (Fernández-Lizarazo & Moreno-Fonseca, 2016a; Jia-Dong et al., 2019; Zou et al., 2021).

Our analysis also revealed an upregulation of several Ethylene Responsive Factor (ERF) genes in the Rhi_h plants (Table 1, Supplementary Table S13). The ethylene-regulated pathway is involved in plant defense responses (van Loon et al., 2006). Interestingly, it has been suggested that ethylene participates in mycorrhizal-induced resistance as well (Jiang et al., 2021; López-Ráez et al., 2010; Pozo et al., 2010). Similar to our finding, in a previous study by Cervantes-Gámez et al. (2016), the authors showed that the majority of mycorrhiza-responsive DEGs observed in tomato, was classified in the category "hormone metabolism", with the sub-category related to ethylene showing the highest number of DEGs. Our observations may thus indicate that the ethylene-regulated pathway is involved in mycorrhiza-induced resistance against *M. sexta* herbivory.

Table 1. Highlighted DEGs from pairwise comparisons between herbivory and non-herbivory treatments in *R. irregularis*-inoculated plants (Rhi_nh vs. Rhi_h) and from *R. irregularis*-inoculated and non-inoculated herbivory-stressed plants (Nm_h vs. Rhi_h). For each DEG, the table includes ITAG4.1 identifier (Gene ID), shrinked Log_2 Fold Change (LFC) and q-value (padj) for each pairwise comparison, along gene symbol and description data.

	Rhi_nh vs Rhi_h		Nm_h vs. Rhi_h			
Gene ID	**LFC**	**padj**	**LFC**	**padj**	**Symbol**	**Description**
Solyc06g035940	8.5	4.0E-8	7.2	5.0E-4	*Sl*ANL2B	Homeobox protein ROC5
Solyc07g150103	7.1	1.0E-5	7.6	8.0E-4		Early nodulin-like
Solyc07g052510	2.0	7.0E-4	-	-		Peroxidase
Solyc10g076240	1.8	2.0E-3	-	-		Peroxidase
Solyc02g062390	5.6	1.6E-14	-	-		Dehydrin
Solyc02g084840	1.3	2.8E-5	-	-		Dehydrin
Solyc01g095080	4.8	2.0E-3	-	-	ACC	ACC synthase
Solyc01g095080	3.3	1.1E-5	-	-	ACC	ACC synthase
Solyc02g084850	2.1	6.8E-7	-	-	TAS14	ABA-inducible dehydrin
Solyc05g051380	1.4	5.0E-3	-	-	AP2/ERF	AP2-like ERF AIL6

Several other stress response genes were exclusively upregulated in Rhi_h plants including two peroxidases, a gene regulating Radical-Induced Cell Death 1 (*RCD1*) and an early nodulin-like gene (Table 1, Supplementary table S4). Exposure to abiotic and biotic stresses results in the formation of reactive oxygen species (ROS) in plants (Babbar et al., 2021; Erb & Reymond, 2019; Kámán-Tóth et al., 2019; Liu et al., 2021; Schwarzländer et al., 2009; Zou et al., 2015). In order to protect themselves against ROS, plants are equipped with ROS-scavenging enzymatic systems comprising of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) (Babajani et al., 2009). Increased peroxidase levels have been shown to enhance plant's ability to tolerate insect feeding (Gulsen et al., 2010; Mai et al., 2016). In addition, the study of Li et al. (2019) demonstrated that mycorrhizal colonization increased peroxidase activity, thereby resulting in higher resistance of alfalfa plants (*Medicago sativa*) against pea aphid (*Acyrtosiphon pisum*) herbivory (Li et al., 2019). Therefore, the upregulation of two genes coding for peroxidases in the Rhi_h plants suggest a mycorrhiza-mediated effect on ROS detoxification in response to *M. sexta* herbivory. ROS production is also able to trigger a signal cascade leading to various outputs including tolerance, acclimatization, and cell death in response to abiotic and biotic stresses (Dat et al., 2000; Mittler, 2002; Vranová et al., 2002). *RCD1* has been shown to control disease responses, cell death, and the meristem fate in a ROS-dependent manner (Cheng et al., 1995; Overmyer et al., 2005; Teotia & Lamb, 2011). Therefore, ROS production, because of *M. sexta* herbivory, probably resulted in the upregulation of *RCD1* in Rhi_h plants.

Furthermore, an early nodulin-like gene was exclusively upregulated in our Rhi_h plants. Genes coding for nodulins were first characterized while studying host plant responses to the development of symbiotic root nodules. More recently, several studies have identified that these genes are also present in non-legume plant species holding key roles in hormone and solute transport during various processes (Denancé et al., 2014). For example, several nodulin genes were found as differentially expressed in the roots of rice (*Oryza sativa*) upon colonization by *Azospirillum brasilense* (Thomas et al., 2019). The upregulation of an early nodulin-like gene in *R*hi_h might thus indicate that this gene plays a role in the hormonal regulation of mycorrhizal plants in response to *M. sexta* herbivory.

Remarkably, an oxidative stress-related peptide methionine (*R*)-sulfoxide reductase protein was downregulated in Rhi_h plants (Table 1, Supplementary table S4). Methionine sulfoxide reductases (MSRs) play protective roles upon abiotic and biotic environmental constraints in plants (Rey & Tarrago, 2018; Rouhier et al., 2006). Furthermore, the involvement of jasmonates in MSR gene expression has been reported in several studies. For instance, in *A. thaliana*, the expression of a MSR gene was strongly induced by linolenic acid, which is a precursor of jasmonic acid (JA) (Mata-Pérez et al., 2015). On the other hand, treatment of tomato seedlings with JA caused either downregulation or no change in the expression levels of four MSR genes (Dai & Wang, 2012). These findings point to a complex regulation of MSR gene expression by jasmonates.

Among herbivory-associated downregulated DEGs, the same enrichment analysis pointed at categories related to “response to auxin”, “response to stimulus”, “protein dimerization activity” and “transferase/hydrolase activity”. It is remarkable that a stronger enrichment in the downregulation of “response to auxin” was observed for Tri plants. This was associated with the downregulation of eight small auxin upregulated RNA (SAUR) genes (Supplementary table S4). *SAURs* belong to a family of auxin-responsive genes present in most of the higher plant species. *Trichoderma* strains are able to produce auxin-related compounds (Contreras-Cornejo et al., 2009). As it has been reported, in *Arabidopsis* plants, *Trichoderma*-produced elicitors facilitate changes in root architecture and increase plant biomass through auxin signaling (Garnica-Vergara et al., 2016). Similarly to our results, the tomato gene *Solyc09g008175.1* that codes for the SAUR-like auxin responsive protein family was downregulated in tomato plants treated with *T. atroviride* P1 (Coppola, Cascone, et al., 2019). Therefore, we hypothesize that inoculation of tomato roots with *T. harzianum* resulted in the upregulation of the auxin signaling pathway that could subsequently lead to an increase in plant biomass in the absence of herbivory. However, it is probable that after *M. sexta* infestation, *T. harzianum*-inoculated plants shifted from growth- to defense-related responses, and this subsequently led to the observed downregulation of *SAUR* genes.

MapMan-based hierarchical clustering over the differentially expressed gene set (LRT, α 0.05) clearly separated herbivory from non-herbivory samples (Figure 2B). Herbivory triggered the downregulation of processes related to photosynthesis, protein biosynthesis, and nutrient uptake. On the contrary, herbivory triggered the upregulation of processes related to stress responses such as polyamine, secondary and amino acid metabolism. Additionally, herbivory led

to stronger upregulation of regulatory- (multi process regulation, chromatin organization) and cell growth- (cell wall, cell cycle, cytoskeleton organization) related categories in herbivore-infested plants (Figure 2B). Interestingly, these shifts were stronger in the microbe-inoculated plants, and particularly in the Tri ones.

We also found a higher upregulation of an anthocyanin 5-aromatic acyltransferase and three type Ⅰ and Ⅱ protease inhibitors in Tri_h plants (Table 2, Supplementary Table S4, S6). Anthocyanins are products of secondary plant metabolism belonging to the chemical group of flavonoids. They play important roles in plant development, such as providing protection against UV radiation, low temperature, drought stress and pathogens (Cao et al., 2016; Ren et al., 2014; Shafiq & Singh, 2018). In accordance with our observation, several studies have shown that *Trichoderma* strains are known to enhance plant secondary metabolism, including anthocyanins (Coppola, Diretto, et al., 2019).

Table 2. Highlighted DEGs from pairwise comparisons between herbivory and non-herbivory treatments in *T. harzianum*-inoculated plants (Tri_nh vs. Tri_h) and from *Trichoderma harzianum*-inoculated and non-inoculated herbivory-stressed plants (Nm_h vs. Tri_h). For each DEG, the table includes ITAG4.1 identifier (Gene ID), shrinked Log_2 Fold Change (LFC) and q-value (padj) for each pairwise comparison, along gene symbol and description data.

	Tri_nh vs Tri_h		**Nm_h vs. Tri_h**			
Gene ID	**LFC**	**padj**	**LFC**	**padj**	**Symbol**	**Description**
Solyc05g047510	5.7	1.0E-4	-	-		Jasmonate sulfotransferase
Solyc12g008740	5.7	4.0E-4	-	-	ACC	ACC synthase
Solyc01g095080	3.1	1.0E-5	-	-	ACC	ACC synthase
Solyc12g042210	2.7	1.0E-3	-	-	AP2/ERF	Ethylene-response factor
Solyc09g089500	-	-	2.2	5.5E-4		Protease inhibitor Ⅰ
Solyc10g081300	-	-	1.9	3.7E-3		Metacaspace-9
Solyc05g052650	1.8	3.0E-3	-	-		Anth. 5-aromatic acyltransferase
Solyc11g021060	-	-	1.6	8.9E-3		Protease inhibitor Ⅱ
Solyc06g034360	-	-	1.6	8.3E-3		Pectineserase
Solyc08g079890	-	-	1.6	7.3E-3	P69Ⅰ	Subtilase (possible phytaspase)
Solyc09g025310	-	-	1.5	5.7E-3	KIRA-1L	NAC TF (Ath KIRA 1 homolog)
Solyc03g098795	-	-	1.0	9.4E-6		Protease inhibitor Ⅱ
Solyc08g078900	-	-	-1.2	3.4E-5	DEA1	Differentially expressed in response to Arachidonic Acid 1

Solyc02g091990	-1.9	3.0E-3	-	-	ACC	ACC synthase
Solyc03g093560	-2.0	1.2E-5	-	-	AP2/ERF	Ethylene-response factor

Inoculation with *T. harzianum* was also associated to changes in signaling mechanisms and herbivory response genes, as pointed by the differential enrichment in "catalytic and transferase activity-related" GO categories, and the upregulation of "secondary/polyamine metabolism" and "multi- process regulation" MapMan categories (Figure 2AB). Among the modulated signaling-related elements, several genes encoding for isoforms of the ethylene synthesis enzyme 1-aminocyclopropane-1-carboxylate synthase (*ACC synthase*) and an APETALA2/ETHYLENE RESPONSIVE FACTOR (AP2/ERF) were differentially modulated in these plants (Table 2, Supplementary Table S4), suggesting a *Trichoderma*-triggered modulation of ethylene signaling under stress. Similar to our findings, two recent studies have suggested a role for ethylene pathway in *Trichoderma*-induced herbivore resistance (Coppola, Cascone, et al., 2019; Coppola, Diretto, et al., 2019).

We further observed the downregulation of the *Differentially Expressed in response to Arachidonic Acid 1* gene (*DEA1*) in Tri_h plants. *DEA1* is a circadian-regulated gene in tomato belonging to the 8-cysteine motif (8CM) proline-rich family proteins (PRPs) (Weyman et al., 2006). DEA1 has been shown as an arachidonic acid-, pathogen-, and JA-inducible gene (Saikia et al., 2020; Weyman et al., 2006). Therefore, the observed suppression of *DEA1* in Tri-h plants could be linked to the modulation of JA levels due to *M. sexta* herbivory.

This analysis also allowed us to identify microbe-associated effects under non-herbivory conditions. The abundance of growth-related categories (protein biosynthesis, cell wall organization) was higher in microbial inoculated plants. We also found a higher regulation of processes related to nutrient uptake in Rhi plants. This is not surprising as colonization by AMF and *Trichoderma* is known to increase the primary metabolism in plants, and the absorption of several nutrients, such as phosphorus and nitrogen (Cervantes-Gámez et al., 2016; Li et al., 2015; Mazzei et al., 2016; Parihar & Bora, 2018; Shoresh et al., 2010; Smith & Read, 2010). This has been linked to a better performance of colonized plants, and a higher tolerance against herbivory (Borowicz, 2013; Contreras-Cornejo, Macías-Rodríguez, et al., 2018; Coppola, Diretto, et al., 2019; Jung et al., 2012; Kula et al., 2005; Shrivastava et al., 2015; Zhou et al., 2018).

3.3 Results from the multivariate analysis over the transcriptomic dataset

We then performed Principal Component Analysis (PCA) over two different subsets of data: the differentially expressed gene set (LRT, α 0.05) including all treatments (Nm, Nm_h, Rhi, Rhi_h, Tri, Tri_h) (Figure 3A) and a dataset including the differentially expressed genes among herbivory samples (Rhi_h, Nm_h, Tri_h) (LRT, α 0.05) (Figure 3B). PCA over the first dataset successfully separated herbivory and non-herbivory samples and grouped herbivory samples by the presence of mutualistic microbes in its first principal component (PC1) (Figure 3A). PC1 was overall

correlated to downregulation of photosynthesis and chloroplastic protein synthesis genes, and to upregulation of stress response genes (protease inhibitors, *LapA1*, arginase) (Supplementary table 8). The second component (PC2) gathered variables associated to the modulation of the plant response by the mutualistic microbes. This component was correlated to chromatin organization and cell cycle/division (cyclins, kinesins) genes, which were further, but not significantly accumulated in Tri_h plants.

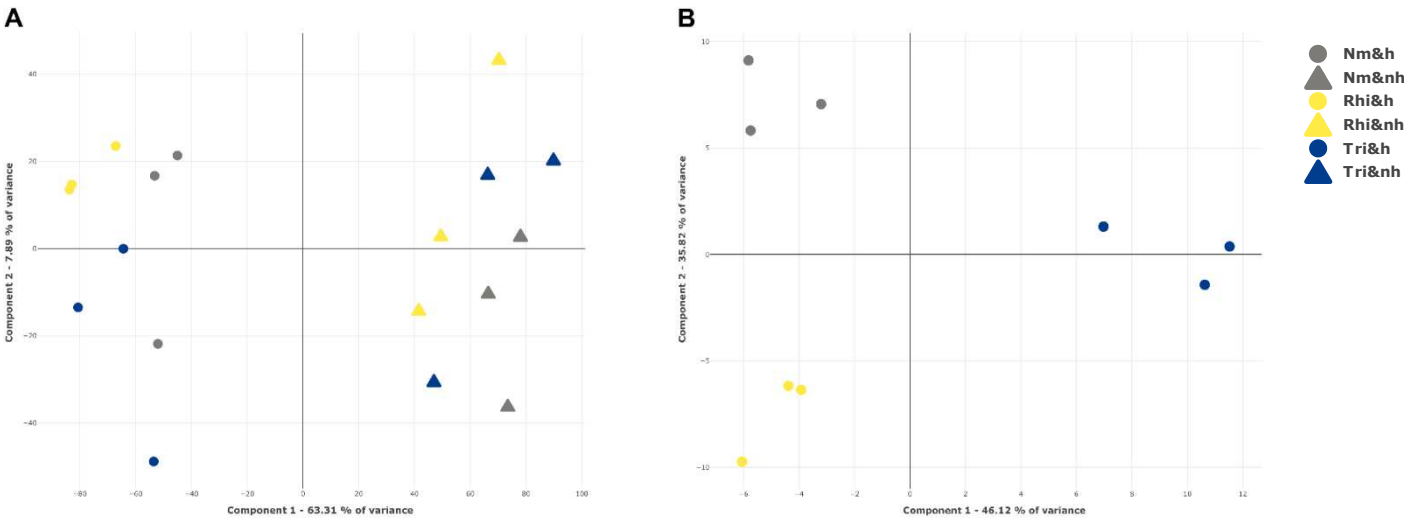

Figure 3. Visualization of the multivariate analysis for the transcriptomic dataset. (A) Principal Component Analysis (PCA) over the differentially expressed genes set including all three treatments. The analysis included the dataset of differentially expressed genes among non-herbivory samples: non-microbe (symbolized as Nm, depicted in grey triangles), *Trichoderma harzianum*-inoculated (symbolized as Tri, depicted in blue triangles) and *Rhizophagus irregularis*-inoculated (symbolized as Rhi, depicted in yellow triangles); and the dataset of differentially expressed genes among herbivory samples: Nm_h-grey dots, Tri_h-blue dots, Rhi_h-yellow dots**. (B)** Principal Component Analysis (PCA) over the differentially expressed genes set among herbivory samples (Nm_h, Tri_h, Rhi_h).

PCA over the "herbivory" dataset separated samples by the presence of mutualistic microbes and by the identity of the mutualistic microbe (Figure 3B). The PC1 explained mostly the Tri modulation of herbivory response, including stress response genes (protease inhibitors, programmed cell death (PCD), secondary metabolism, signaling, and cell wall-related genes), whose expression was significantly different in Tri_h plants (Table 2, Supplementary table 10). In response to various abiotic and biotic stresses, plants undergo PCD. PCD depends on the outcome of several biochemical events that occur in response to stress, such as the generation of ROS, ionic influx/efflux, biosynthesis of phytohormones, phytoalexins and polyamines (Prasad et al., 2022). Interestingly, *Trichoderma* strains have been reported to induce PCD to fungal phytopathogens through the production of antimicrobial compounds, such as peptaibols (Shi et

al., 2012; Tijerino et al., 2011). The PC2 explained mostly the Rhi modulation of herbivory response including genes such as the early nodulin-like protein 1 and the transcription factor AP2/B3 (Table 1, Supplementary table 6).

3.4 Metabolomics dataset description and results from the multivariate analysis

Metabolomics dataset generated over the same samples included in total 2,920 signals with 464 showing significant intensity differences between treatments (ANOVA, α 0.05) (Supplementary table S5). The multivariate analysis over the metabolomics dataset revealed a clear separation between herbivory and non-herbivory samples, as occurred with the transcriptomic dataset (Figure 4). Herbivory and non-herbivory samples were separated along PC1. Interestingly, the PC2 separated the samples in relation to the microbial treatment. This separation was more evident than in the case of the transcriptomic dataset (Figure 4).

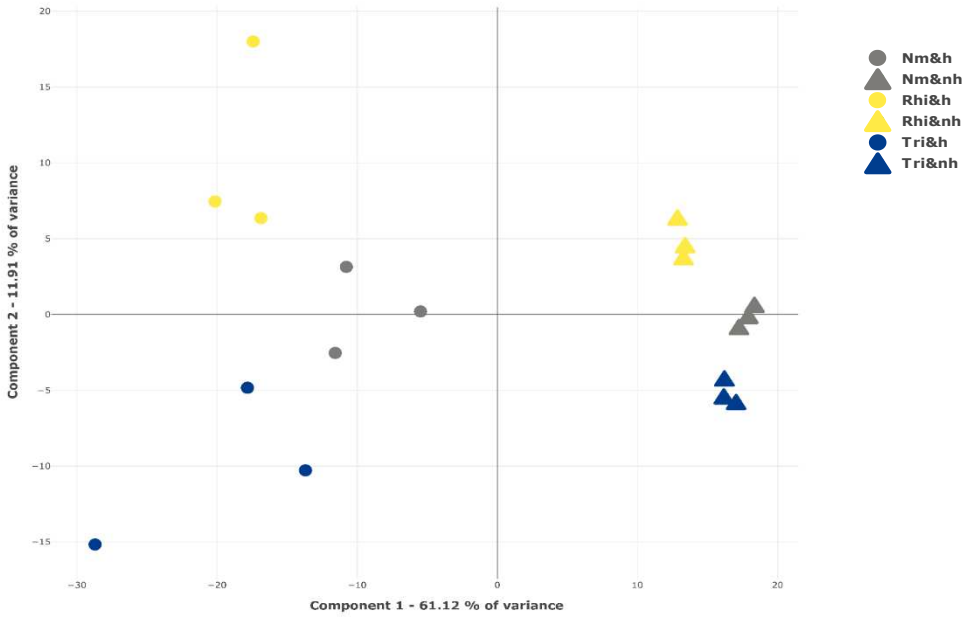

Figure 4. Visualization of the multivariate analysis for the metabolomic dataset. Principal Component Analysis (PCA) over herbivory vs. non-herbivory samples for all three treatments. The analysis included non-herbivory samples: non-microbe (symbolized as Nm, depicted in grey triangles) *Trichoderma harzianum*-inoculated (symbolized as Tri, depicted in blue triangles) and *Rhizophagus irregularis*-inoculated (symbolized as Rhi, depicted in yellow triangles) and herbivory samples including Nm_h (grey dots), Tri_h (blue dots) and Rhi_h (yellow dots).

3.5 Results from the transcriptomic and metabolomics integrative analysis

Integrative sparse Partial Least Squares (sPLS) analysis over the top 500 variables within the differentially expressed gene and metabolite sets (LRT, α 0.05; ANOVA, α 0.05) (used as prediction and predicted matrices, respectively) successfully split samples by herbivory treatment in Principal Component 1 (Figure 5A). Notably, Principal Component 2 mostly separated the samples depending on the microbial treatment.

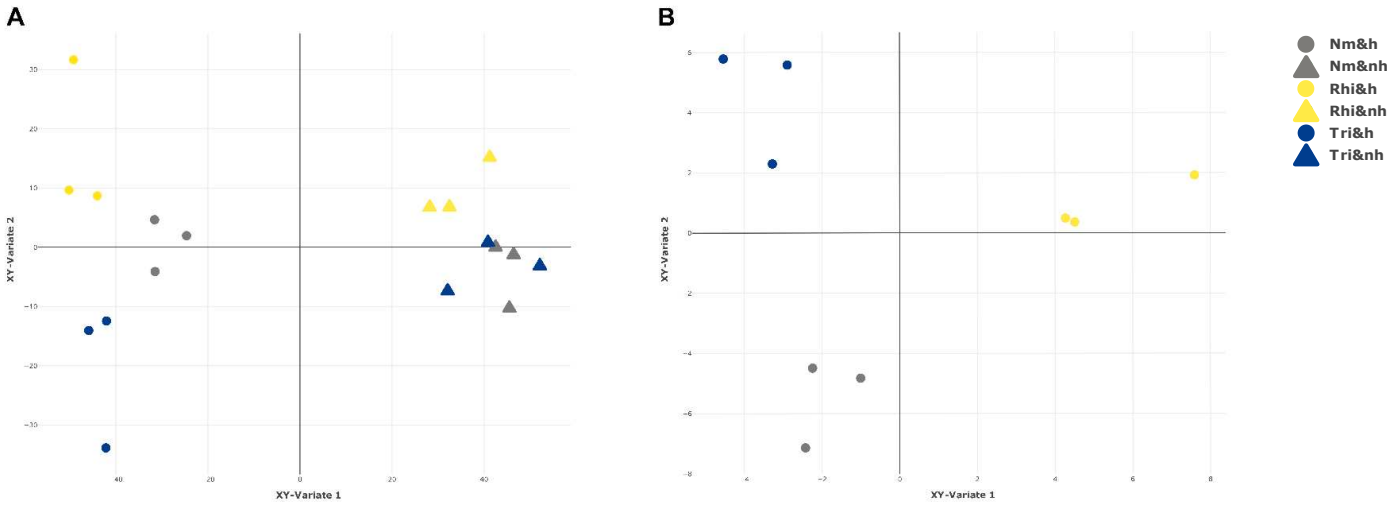

Figure 5. Visualization of the transcriptomic and metabolomic integrative analysis. (A) sparse Partial Least Squares discriminant analysis (sPLS) analysis over the differentially expressed genes and metabolites sets for all three treatments (non-inoculated-Nm, *Trichoderma harzianum*-inoculated-Tri and *Rhizophagus irregularis*-inoculated-Rhi) with and without herbivory. **(B)** sPLS analysis exclusively within herbivory samples Nm_h (grey dots), Tri_h (blue dots), Rhi_h (yellow dots).

Following the global sPLS analysis, we performed a second sPLS-based integration approach over the herbivory dataset. This analysis successfully separated our samples by the presence of mutualistic microbes and by the species of these mutualists (Figure 5B). Subsequently, we performed a correlation network analysis over this data set. As a result, we generated two correlation sub-networks enriched in elements modulated by the presence of Tri or Rhi, respectively (Figure 6). The Tri-focused network summarized the modulation of herbivory response due to the presence of Tri fungus, placing the metabolite signals 886, 1508 and 2551 at its center. This network depicts the modulation of programmed cell death (PCD) showed by the enhancement of *KIRA1* homolog (*KIRA1-L*), Metacaspase-9, and P69I subtilisin. In addition, this modulation was concomitant with the upregulation of protease inhibitors and cell wall remodeling elements such as pectinesterase (Figure 6A, Table 2, Supplementary table S4, S6, S11). Rhi-focused herbivory sub-network expands around the 2922 metabolite signal, which is

differentially accumulated in Rhi_h plants (Figure 6B). This metabolite signal was positively correlated to auxin-related elements such as the *IAA36* (*Solyc06g066020*) and *Big grain 1-like A* (*Solyc08g062180*) genes. The central metabolite signal was also positively correlated to ribosome maturation and sulphate transport, but negatively correlated to the transformation of phosphatydiletanolamine to phosphatidylcholine (Figure 6B).

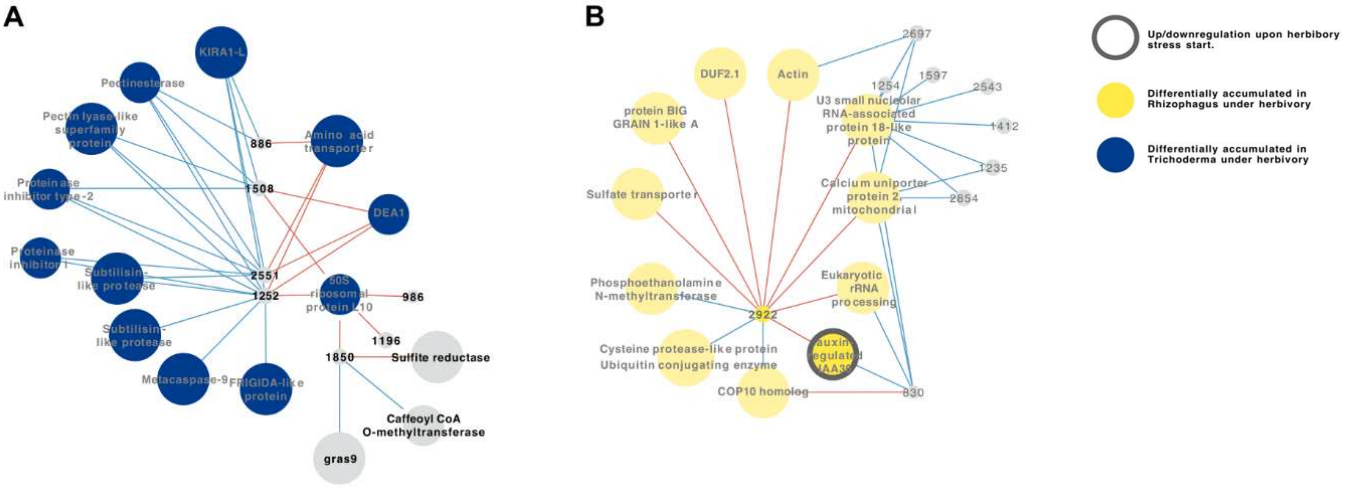

Figure 6. Visualization of the correlation network analysis within the herbivory dataset. (A) Correlation subnetwork analysis enriched for elements modulated by the presence of *Trichoderma harzianum* (Tri) depicted in blue nodes. **(B)** Correlation subnetwork analysis enriched for elements modulated by the presence of *Rhizophagus irregularis* (Rhi) depicted in yellow nodes. The nodes depicted in grey color represent elements that were enriched in both microbial treatments independently of the microbial identity.

4. Conclusions

The transcriptomic analysis showed that in the absence of herbivory, microbial-inoculated plants exhibited enhanced levels of growth-related categories, such as protein biosynthesis and cell wall organization. Remarkably, nutrient uptake was higher regulated in the Rhi plants. PCA over herbivory vs. non-herbivory datasets, successfully separated herbivory samples from the non-herbivory ones in PC1. This component was overall correlated to: ⅰ) downregulation of photosynthesis and cholroplastic protein synthesis genes, and ⅱ) upregulation of stress-responsive genes, as for instance, protease inhibitors. On the other hand, PC2 gathered variables associated specifically with the microbial-triggered modulation of tomato plant defenses to herbivory. The PCA performed exclusively over the herbivory samples dataset separated the samples by the presence of mutualistic microbes, and by the identity of each microbe, as well.

On one hand, PC1 explained mostly the Tri-triggered modulation of tomato plant defenses against *M. sexta* herbivory, including stress-responsive genes (PIs, PCD, secondary metabolism, signaling, and cell wall-related genes). On the other hand, PC2 explained the Rhi-tirggered modulation of tomato defenses to *M. sexta* herbivory, gathering genes such as two peroxidases, an early nodulin-like gene, a gene regulating RCD, and the TF AP2/B3.

In the multivariate analysis conducted over the metabolomics dataset, a clear separation was displayed between herbivory and non-herbivory samples, as happened with the transcriptomic analysis. Herbivory and non-herbivory samples were separated along PC1, and PC2 separated the samples depending on the mutualistic microbe. Furthermore, the integrative sPLS analysis performed within DEGs and the dataset of metabolites successfully split the samples by herbivory in PC1. On the other hand, PC2 separated the samples in respect to the mutualistic microbe. Narrowing down our analysis, the sPLS-based integration performed exclusively over the herbivory dataset successfully split the samples according to the mutualistic microbes. The correlation network analysis over the same dataset revealed two correlation sub-networks, Rhi and Tri, enriched in elements modulated by the presence of mycorrhiza and *Trichoderma*, respectively.

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Root symbionts alter herbivore-induced indirect defenses of tomato plants by enhancing predator attraction

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Beneficial root microbes are among the most frequently used biocontrol agents in cropping systems, since they have been shown to promote plant growth and crop yield. Moreover, they are able to enhance protection against pathogens and insect herbivores by activating plant resistance mechanisms. Plant defense responses against herbivorous insects include the induction of metabolic pathways involved in the synthesis of defense-related metabolites. These metabolites include volatile organic compounds (VOCs), which attract natural enemies of the herbivores as a form of indirect resistance. Considering that beneficial root microbes may affect direct herbivore resistance, we hypothesized that also indirect resistance may be affected. We tested this hypothesis in a study system composed of tomato, the arbuscular mycorrhizal fungus *Rhizophagus irregularis*, the growth-promoting fungus *Trichoderma harzianum*, the generalist chewing herbivore *Spodoptera exigua* and the omnivorous predator *Macrolophus pygmaeus*. Using a Y-tube olfactometer we found that *M. pygmaeus* preferred plants with *S. exigua* herbivory, but microbe-inoculated plants more than non-inoculated ones. We used a targeted GC-MS approach to assess the impact of beneficial microbes on the emission of volatiles 24 h after herbivory to explain the choice of *M. pygmaeus*. We observed that the volatile composition of the herbivore-infested plants differed from that of the non-infested plants, which was driven by the higher emission of green leaf volatile compounds, methyl salicylate, and several monoterpenes and sesquiterpenes. Inoculation with microbes had only a marginal effect on the emission of some terpenoids in our experiment. Gene expression analysis showed that the marker genes involved in the jasmonic and salicylic acid pathways were differentially expressed in the microbe-inoculated plants after herbivory. Our results pinpoint the role of root symbionts in determining plant-microbe-insect interactions up to the third trophic level, and elucidates their potential to be used in plant protection.

KEYWORDS

arbuscular mycorrhizal fungi, *Trichoderma*, *Spodoptera exigua*, *Macrolophus pygmaeus*, GC-MS, herbivore-induced plant volatiles, multi-trophic interactions

Introduction

Plants encounter multiple biotic and abiotic challenges simultaneously in their habitats (Walling, 2000). Among the biotic challenges they face, insect pests represent one of the most important factors, leading to extensive agricultural crop losses. According to recent studies, on average, pests can destroy up to 40% of global crop production (Savary et al., 2019; IPPC Secretariat, 2021). To counter herbivore attack, plants mount a wide array of defense mechanisms, which include specialized morphological structures or secondary metabolites and proteins that have toxic, repellent and/or anti-nutritional effects on herbivores (Usha Rani and Jyothsna, 2010; War et al., 2012). Defense mechanisms that affect host plant preference, survival or reproductive success are characterized as direct defenses, whereas mechanisms that involve natural enemies of the attacking pests are characterized as indirect defenses (Turlings and Erb, 2018). One form of indirect plant defenses in response to insect herbivory is the release of a bouquet of Herbivore-Induced Plant Volatiles (HIPVs) that specifically attract natural enemies of the attacking herbivores (Arimura et al., 2009; McCormick et al., 2012). The blends of HIPVs emitted in response to herbivore attack predominantly include terpenes, green leaf volatiles (GLVs) and methyl salicylate (MeSA) (Dudareva et al., 2006; Maffei, 2010). HIPV emission results from the activation of specific genes, which are involved mainly into the terpenoid, lipoxygenase, and shikimic acid pathways (Dudareva et al., 2006; Pichersky et al., 2006; Maffei, 2010). These so-called info-chemicals serve several roles, such as the interaction of plants with arthropods, microorganisms, undamaged neighboring plants, or intra-plant signaling that warns undamaged parts within the plant under attack (Karban, 2011; Turlings and Erb, 2018). Therefore, HIPVs can be effectively used in crop pest management.

In the last decades, management of crop pests has heavily relied on conventional insecticides resulting in reduced crop losses by pests. However, the intensive and large-scale application of insecticides has caused several adverse effects on the environment. Persistent pesticides are found in soils and water, which affects not only target but also of non-target species (Bragança et al., 2018). Moreover, the adaptation and increased resistance to pesticides developed by the target pests has led to the need of using higher amounts and new chemical compounds to protect crops every year, leading to undesired side effects and increasing the costs of food production (Carvalho, 2006). Integrated Pest Management (IPM) is an effective, environmentally sound approach to pest management (Kabir and Rainis, 2015). IPM strategies aim at protecting air, water and soil resources and employ a variety of pest-control methods in a way that facilitates biological control of insect pests in order to improve economic, public health and environmental outcomes (Alam et al., 2016).

In their habitats, plants not only have detrimental but also symbiotic interactions with several growth promoting microbes. Such microbes provide plants with an improved nutritional status (Hodge and Fitter, 2010; Samolski et al., 2012); tolerance to biotic and abiotic stresses (Fontenelle et al., 2011; Degola et al., 2015) and improved fruit yield and quality (Hermosa et al., 2012; Bona et al., 2018). In the last decades, root beneficial microbes have also been included in IPM programs in an effort to enhance the efficacy of pest control, reduce the use of synthetic chemicals, increase crop yield and improve the quality of products (Woo et al., 2014).

A well-documented example of plant-associated beneficial microbes are the arbuscular mycorrhizal fungi (AMF). AMF are symbiotic inhabitants of plant roots and form associations with over 80% of the terrestrial plant species (Parniske, 2008). They are able to enhance plant growth and development by improving the acquisition of water and mineral nutrients, such as inorganic phosphate and various micronutrients (Smith and Smith, 2011; Hodge and Storer, 2015). Moreover, mycorrhizal plants have shown higher tolerance to abiotic stress agents (Miransari, 2010; Smith et al., 2010; Orine et al., 2022) and increased resistance to plant pests and diseases through the induction of systemic resistance (Pozo and Azcón-Aguilar, 2007; Pineda et al., 2010; Jung et al., 2012). In exchange, AMF obtain plant-derived photoassimilates (Bago et al., 2000; Smith and Read, 2010) produced during photosynthesis. Although the fungal structures formed during mycorrhizal symbiosis are restricted in the plant roots, AMF trigger systemic biochemical changes in the composition and concentration of primary and secondary metabolites in the aboveground plant tissues (Schweiger et al., 2014; Schweiger and Müller, 2015). Such metabolic changes influence not only the performance of the insect herbivores feeding on the host plants (Papantoniou et al., 2021), but also their interactions with their natural enemies (Minton et al., 2016). Studies have shown that arbuscular mycorrhizal fungi can modulate the indirect plant defenses and the recruitment of natural enemies of herbivorous pests as well (Fontana et al., 2009; Schausberger et al., 2012; Babikova et al., 2014).

In the broad spectrum of plant-associated microbes, various strains of the soil-borne fungus *Trichoderma* have direct beneficial effects on plants including the promotion of growth and nutrient uptake, better assimilation of nitrogen and enhancement of plant defenses against abiotic and biotic stress factors (Shoresh et al., 2010; Studholme et al., 2013; Lorito and Woo, 2015). *Trichoderma* strains have been reported to alter herbivore-induced plant volatiles and the expression levels of genes involved in the induction of indirect

defense responses resulting in a stronger attraction of natural enemies towards the herbivorous pests (Battaglia et al., 2013; Coppola et al., 2017; Coppola et al., 2019).

Tomato (*Solanum lycopersicum* L.) is the second most cultivated vegetable crop throughout the world and susceptible to numerous pests that can severely affect its nutritional value and taste (Zhang et al., 2021). Among them are the larval stages of *Spodoptera exigua* (Hübner), the beet armyworm, which is a polyphagous insect with a wide global distribution (Moulton et al., 2000; Wang et al., 2006; Zheng et al., 2011). Therefore, IPM strategies combining the use of root symbionts and natural enemies to prey on *S. exigua* larvae could be a promising approach to control tomato pests in greenhouse and field crops.

Hemipteran predators of the Miridae family are commonly used as natural enemies to control pests. Recent studies suggest that these mirid predators use HIPVs as cues to locate their prey (Moayeri et al., 2007; Lins et al., 2014; Silva et al., 2018). Use of such info-chemicals has been observed among many predaceous arthropods independently of their diet specialization (Dicke and Sabelis, 1988); however, learning to respond to info-chemicals occurs more commonly in generalists than in specialists (Steidle and Van Loon, 2003). The zoophytophagous mirid *Macrolophus pygmaeus* Rambur (Hemiptera: Miridae) is broadly used as a biocontrol agent to control greenhouse and field tomato pests in Europe (Castañé et al., 2004; Perdikis et al., 2008; Arnó et al., 2010). The predator is mass-reared and released to control thrips, whiteflies, mites and lepidopterans, but can also prey on many other pest species (Van Lenteren et al., 2020). *M. pygmaeus* reacts to volatiles emitted by prey-infested plants, but not to volatiles emitted directly by the prey (Moayeri H. et al., 2006; Moayeri H. R. et al., 2006; Ingegno et al., 2011; Mollá et al., 2014).

So far, few studies focusing on the effect of root symbionts on the induction of indirect defenses of tomato plants against herbivores and the attraction of their natural enemies have been conducted. The majority has cast light on the effect of tomato root-associated microbes on the emission of volatiles after aphid infestation and their contribution to attracting aphid predators or parasitoids (Guerrieri et al., 2004; Battaglia et al., 2013; Coppola et al., 2017). Only the study of (Coppola et al., 2019) investigated how the biocontrol agent *Trichoderma atroviride* strain P1 induces tomato plant responses against two insects with different feeding habits, the noctuid caterpillar *Spodoptera littoralis* and the aphid *Macrosiphum euphorbiae*. They found that *T. atroviride* P1 altered the plant metabolic pathways leading to the production and emission of VOCs, which were linked to the higher attraction of the parasitoid *Aphidius ervi*. This indicated that root symbionts might affect the response of natural enemies to the plant. However, they tested the attraction of the parasitoid, toward *T. atroviride* P1-inoculated and untreated plants without prior exposure to the herbivores and then collected the volatiles from the plants used in the wind-tunnel bioassays.

To widen our knowledge of the effect of root symbionts on indirect defenses, we set up a series of experiments with tomato plants root-inoculated with two different beneficial microbes, the arbuscular mycorrhizal fungus *Rhizophagus irregularis* or the plant growth-promoting fungus *Trichoderma harzianum*. We exposed the plants to herbivory by the generalist lepidopteran herbivore *S. exigua* and assessed the choice behavior of the zoophytophagous mirid predator *M. pygmaeus*. We hypothesized that inoculation of plants with root symbionts would enhance the indirect defense responses of tomato in response to herbivory, resulting in higher expression of the genes involved in the biosynthesis of herbivore-induced plant volatiles and therefore, a stronger attraction of *M. pygmaeus*. To test our hypotheses, we used a Y-tube olfactometer to test the preference of *M. pygmaeus* towards microbe-inoculated and non-inoculated plants with and without the herbivore. To identify potential mechanisms driving *M. pygmaeus* preferences, we collected volatiles emitted by non-inoculated and microbe-inoculated plants in the presence and absence of aboveground herbivory. We complemented our study with gene expression analysis of specific genes, which are involved in the biosynthetic pathways that regulate the synthesis of herbivore-induced plant volatiles. These complementary analyses allowed us to generate some more insights in the interactions between root symbiotic fungi and natural control agents commonly used in tomato culture.

Materials and methods

Plant and fungal material

Tomato (*Solanum lycopersicum*) cultivar Moneymaker was used in all the bioassays as well as in the predator rearing. Tomato seeds were purchased from Intratuin B.V (Woerden, Netherlands). *Rhizophagus irregularis* was used as the AM fungus. The *R. irregularis* solid inoculum (DAOM197198 research grade, 1 million spores in 100 g attapulgite powder, batch S.380—02.2021) and the heat-sterilized carrier material were purchased from SYMPLANTA GmbH & Co. KG (Darmstadt, Germany, https://www.symplanta.com) and stored at 8°C until used. *Trichoderma harzianum* isolate T-78 (CECT 20714, Spanish Type Culture Collection) was routinely cultured on potato dextrose agar (PDA, Sifin Diagnostics, Berlin, Germany) plates and regularly sub-cultured. The *T. harzianum* inoculum was prepared on a solid medium containing commercial oat and vermiculite according to Martínez-Medina et al. (2009).

Insect rearing

Eggs of *S. exigua* (Lepidoptera, Noctuidae) were obtained from Entocare Biologische Gewasbescherming (Wageningen,

Netherlands, www.entocare.nl). The colony of *S. exigua* was routinely maintained in a growth chamber (E-36L, Percival Scientific, Perry, United States) at 25°C, 12 h L, and 12 h D, 45% RH conditions and fed upon artificial diet (Hoffman et al., 1966). Three hundred adults of *M. pygmaeus* were purchased from Katz Biotech AG (Baruth, Germany) and reared in the laboratory reaching adult developmental stage in the next generation. The insects were reared in net cages containing young tomato plants that were grown exclusively for this purpose. The omnivorous insects were provided *ad libitum* with supplementary food including *Sitotroga* eggs (Katz Biotech AG, Baruth, Germany), 1:4 diluted honey water (v/v) soaked on a piece of cotton pad, and organic bee pollen (Biojoy GmbH, Nürnberg, Germany) as described by Vandekerkhove and De Clercq (2010). The insect cages were placed in a plant growth chamber (E-36L, Percival Scientific, Perry, United States) at 24°C with 14 h L and at 21°C with 10 h D and 60% RH conditions. The plants used for *M. pygmaeus* rearing in the net cage were provided with tap water every 2 days and fertilized with Hoagland nutrient solution (Hoagland and Arnon, 1950) once per week.

Plant growth conditions and fungal inoculation

Tomato seeds were surface-sterilized with 40 ml of 10% (v/v) sodium hypochlorite (NaOCL, 12% ChemSolute, Th. Geyer, Berlin, Germany) for 3 min, then thoroughly rinsed in 40 ml of warm tap water repeatedly for four times. After sterilization, the seeds were placed on fine-grained, moist vermiculite and germinated in the dark for 3 days at 28°C. Subsequently, the germinated seedlings were placed in a plant growth chamber (E-36L, Percival Scientific, Perry, United States) at 24 ± 3°C, 14 h/10 h L/D and 60–65% RH conditions. Ten days after germination, the seedlings were transplanted into 1 L pots containing a sterile soil/sand mixture (1:1, v/v). Inoculation with *R. irregularis* was achieved by applying 30 AMF spores per 1 ml of sterile potting substrate mixture directly to the root while potting in order to achieve higher initial concentrations than homogenizing the soil with the inoculum. Inoculation with *T. harzianum* was achieved by mixing the *T. harzianum* inoculum with the sterile potting substrate to achieve a final density of 1×10^6 conidia g^{-1} before transplanting (Martínez-Medina et al., 2017). The plants were then placed in a greenhouse (3.8 m × 6 m) in a completely randomized design with supplemental LED lighting of 3500 k and 80 CRI (RUBOL JOSEPHINE 135W V2 LUMINUS CXM-32 DIY KIT, Rubol, Dronten, Netherlands), 16 h/8 h L/D and ventilation provided within 10 min intervals. The air temperature and relative humidity in the greenhouse were recorded while the experiment took place. The recorded conditions during day and night were 25.82 ± 3.79°C with 46.87 ± 4.76% RH and 22.29 ± 2.84°C with 49.29 ± 4.19% RH, respectively. The pots were bottom watered *via* separate plant saucers every second day with 50 ml tap water; and once a week with half-strength Hoagland nutrient solution (Hoagland and Arnon, 1950). The position of pots was rotated on a week basis to avoid spatial effects inside the greenhouse.

Rhizophagus irregularis and *Trichoderma harzianum* root colonization

The presence *R. irregularis* in the roots of tomato plants was confirmed by incubating washed roots in 10% pre-heated KOH (≥85% p.a., ROTH, Karlsruhe, Germany) for 60 min. Subsequently, the fungal structures were stained with Trypan blue (ROTH) for 10 min at 80°C (Phillips and Hayman, 1970). Approximately 30 1-cm-long root segments were cut from each root sample to be observed under the microscope. The intensity of mycorrhizal structures was evaluated using a five-class system described by Trouvelot et al. (1986) under a binocular stereo microscope (Leica DM 4000 B LED). The quantification of mycorrhizal colonization in the root system (M%) was determined using the method of Mycocalc (https://www2.dijon.inra.fr/mychintec/Mycocalc-prg/download.html) (Supplementary Table S2). Similarly, the root samples of non-inoculated and *T. harzianum*-inoculated plants were also stained and microscopically observed, in order to confirm the absence of any mycorrhizal structures. Non-inoculated and *T. harzianum*-inoculated plants, in the roots of which mycorrhizal structures were observed, were excluded from analyses. The presence of *T. harzianum* in the soil samples of *T. harzianum*-inoculated plants was confirmed by using the plate count technique on PDA plates amended with 50 mg L^{-1} rose bengal (Applichem, Darmstadt, Germany) and 100 mg L^{-1} streptomycin sulphate (ROTH) (Martínez-Medina et al., 2009). The plates were incubated at 28°C in darkness, and colony forming units (CFUs) were counted 5 days later. Soil from non-inoculated and *R. irregularis*-inoculated plants was also sampled, in order to confirm the absence of *T. harzianum* (Supplementary Table S3). In case that *T. harzianum* CFUs had grown on PDA plates containing soil from non-inoculated and *R. irregularis*-inoculated plants, these plants were excluded from analyses.

Y-tube olfactometer bioassays

To assess the olfactory response of *M. pygmaeus* toward the volatile blends emitted by differently root-inoculated plants in the presence and absence of *S. exigua* herbivory for 24 h, we performed a series of vertical Y-tube olfactometer assays (Takabayashi and Dicke, 1992). The Y-tube olfactometer (18 mm diameter, main arm 14 cm long, side arms 10 cm, 110° angle between the side arms) was connected to a pressurized air generation and flow system. Each side arm of

the olfactometer was connected to a 1.5 L glass vessel containing one intact tomato plant. The air was generated by an air compressor (OLF2502, Jenpneumatic und Schlauchtechnik GmBh, Germany). The pressurized air was purified by passing through a glass bottle, humidified and entered into the odor chamber at a rate of 500 ± 50 ml/min regulated by a flowmeter. The airflow was measured with two flow sensors (PFMV510-1, SMC, Japan) adjusted next to each side arm of the Y-tube. Each pot was wrapped in aluminum foil to prevent the collection of volatiles emitted by soil or plastic. Plastic tubing was used for the connections between the different compartments of the set-up. To exclude visual cues and spatial effects, the bioassay took place in a darkroom. Additional lighting (LEICA KL1600 LED, the 4th level) was provided at the end of each side arm of the Y-tube, and the odor chambers were screened off.

A single female predator (4th to 5th instar) was introduced into the main arm of the olfactometer and allowed to make a choice between the two arms (volatile sources). Each female was considered to have made a choice when walking at least 4 cm inside the chosen arm. Females that made no choice within 10 min were excluded from analysis. Each predator was used only once in the olfactometer bioassays and had no visual contact with the plants. At least 4 h before the bioassays, the predators were isolated from the colony and placed individually in a plastic container with perforated lid in a dark room for starvation. We recorded 40—50 replicates (individuals) depending on the treatment combination. After testing five insect individuals, the position of the two odor source chambers was switched between right and left side olfactometer arms in order to avoid positional effects. After every set of ten observations, the Y-tube and the odor chambers were thoroughly washed with ethanol 70% and allowed to dry in a drying oven, before being re-used. The bioassays were performed in a laboratory at 24 ± 2°C and 60%–70% RH from 10:30 to 18:00. Predator responses were assessed for all combinations of the following treatments: 1) non-inoculated plants (Nm)- clean air, 2) non-inoculated plants (Nm)- non-inoculated *S. exigua*-infested plants (non-inoculated + h), 3) non-inoculated *S. exigua*-infested plants (Nm + h) - *R. irregularis*-inoculated *S. exigua*-infested plants (*Rhi* + h), 4) non-inoculated *S. exigua*-infested plants (Nm + h)- *T. harzianum*-inoculated *S. exigua*-infested plants (*Th* + h), 5) *R. irregularis*-inoculated *S. exigua*-infested plants (*Rhi* + h) - *T. harzianum*-inoculated *S. exigua*-infested plants (*Th* + h). All the tests were conducted in a random order to avoid any temporal effects.

Impact of *Rhizophagus irregularis* and *Trichoderma harzianum* on the emission of *Spodoptera exigua* herbivory-induced volatiles

One day prior to herbivory bioassays, second- and third-instar *S. exigua* larvae were removed from the artificial diet and let to feed upon detached tomato leaves for acclimatization overnight. During acclimatization, the larvae were placed in the greenhouse next to the experimental chamber. The day after, three third-instar *S. exigua* larvae were placed on the apical leaflet of the third fully expanded leaf (counting from above) for 24 h. We used clip cages to confine the herbivores to one leaf. To control for potential effects of having a clip cage, the apical leaflet of the third fully expanded leaf of plants without herbivores was also enclosed into a clip cage for 24 h. After 24 h, the herbivores were removed and the volatile collection followed.

Volatile collection and analysis

To investigate the effect of beneficial microbes on the emission of herbivore-induced volatiles, we collected the volatiles emitted by both herbivore- and non-herbivore-infested plants in the greenhouse. Volatiles were collected from Nm (non-inoculated, n = 6), Nm + h (non-inoculated + *S. exigua*, n = 7), *Rhi* (*R. irregularis*-inoculated, n = 7), *Rhi* + h (*R. irregularis* + *S. exigua*, n = 7), *Th* (*T. harzianum*-inoculated, n = 7) and *Th* + h (*T. harzianum* + *S. exigua*, n = 7) plants. The collection of volatiles took place from 10:00 to 14:00; the temperature was 25.61 ± 1.19 °C and 42.27 ± 2.09% RH. We followed the passive trapping method described by Kallenbach et al. (2015) using polydimethysiloxane (PDMS) tubes. Metallic wire cuttings were mounted on the clip cage adjusted at every experimental plant. Two PDMS tubes were used as technical replicates in each clip cage. After 24 h of *S. exigua* herbivory, the tubes were left to collect volatiles for 4 h. The background air volatiles (blank samples) were also collected using the same set-up at each corner of the greenhouse in the absence of plants. When the passive trapping was completed, each pair of PDMS tubes was stored into individually labelled 4 ml glass vials, tightly closed with a screw cap and immediately kept at -20°C until analyzed.

Subsequently, one technical replicate from each PDMS tube pair that was used to collect volatiles was transferred into an empty stainless-steel tube (MARKES, Llantrisant, UK), and the samples were analyzed using a Thermal Desorption-Gas Chromatograph—Mass Spectrometer (TD-GC-MS). TD-GC-MS consisted of a thermodesorption unit (MARKES, Unity 2, Llantrisant, UK), equipped with an autosampler (MARKES, Ultra 50/50), and a gas chromatograph (Bruker, GC-456, Bremen, Germany) connected to a triple-quad mass spectrometer (Bruker, SCION, Hamburg, Germany). The tubes were desorbed following the conditions described below: Dry purge 5 min at 20 ml/min, pre-purge 1 min (or 2 min) at 10 ml/min to remove remaining water, desorption 8 min at 200°C with 60 ml/min, trap temperature 0°C, pre-trap fire purge 1 min at 60 ml/min, split flow 20 ml/min, trap heated to 230°C at

maximum rate and hold for 4 min. The separation of compounds took place on a DB-WAX column (30 m × 0.25 mm inner diameter x 0.25 µm film thickness, manufactured by Restek (Bellefonte, PA, United States, distributed by Analytik, Bad Homburg, Germany). The conditions for GC were set as following: 60°C [hold 1 min 30°C/min to 150°C, 10°C/min to 200°C and 30°C/min to 230°C (hold 5 min)]. Helium was used a carrier gas with a constant flow of 1 ml/min. The mass spectrometry conditions were set at 40°C for the manifold, 240°C at the transfer line, and 220°C for the ion source. The scan range was 33–500 m/z for a full scan, and the scan time was 250 ms (Sam et al., 2021).

A large body of scientific studies have showed that the volatile blends emitted by tomato plants after herbivory include compounds belonging to the chemical classes of green leaf volatiles (GLVs), terpenoids and the aromatic compound methyl salicylate (MeSA) (Ament et al., 2004; Sasso et al., 2007; Battaglia et al., 2013; Coppola et al., 2017; Pappas et al., 2018). Therefore, we followed a targeted volatile analysis approach to annotate compounds belonging to these chemical classes. For our analysis, we selected the most prominent peaks in the chromatograms (signal to noise ratio >10), that could be assigned to GLVs, monoterpenes and sesquiterpenes and methyl salicylate. That resulted in 18 peaks. The compounds were annotated with spectral libraries (National Institute of Standards and Technology (NIST 20), Wiley 20) and compared to retention indices from the literature. When possible, peaks were additionally identified by injection of authentic standards.

Extraction of tomato leaf total RNA and cDNA synthesis

After completing the collection of volatile compounds, the leaflets enclosed into the clip cages from both *S. exigua*-infested and non-infested plants of all treatments were harvested, flash-frozen in liquid nitrogen, according to Bandoly and Steppuhn (2016), and stored at −80°C for further analyses. To explore the effects of root symbionts on indirect defense responses of tomato in response to *S. exigua* herbivory, we measured the expression levels of two jasmonic acid (JA) pathway-related genes: the lipoxygenases *LOX* and *LOXA*, one allene oxide synthase 2 (*AOS2*) and one terpene synthase gene (*TPS5*). *LOX*, *LOXA* and *AOS2*, which are components of octadecanoid signal transduction pathway, are involved in the biosynthesis of JA and green leaf volatiles (GLVs); and *TPS5* contributes in the biosynthesis of the monoterpene linalool (Cao et al., 2014). Among the SA-pathway-related genes, we selected the genes phenylalanine ammonia-lyase (*PAL*) and the salicylic acid carboxyl methyltransferase (*SAMT*). In the phenylpropanoids pathway, *PAL* is involved into the salicylic acid (SA) biosynthesis and *SAMT* modifies SA into methyl-salicylate (MeSA), which is volatile (Ament et al., 2004). RNA was extracted from leaf tissues according to Oñate-Sánchez and Vicente-Carbajosa (2008) with slight modifications. The samples were treated with DNAse I (Thermo Scientific) and cDNA was synthesized with a 1st-strand cDNA synthesis kit (Thermo Scientific) as described in the protocol. Quantitative PCR was conducted using the SYBR® Green Supermix (Bio-RAD, Germany) following the manufacturer's instructions and a thermal cycler (Bio-RAD, C1000 Touch Thermal Cycler CFX 384, Germany) with the following cycling program: 2 min 50°C and 10 min 95°C, 40 cycles of 15 sec 95°C and 1 min 60°C, followed by a melting curve analysis. The sequences of the gene-specific primers used in qPCR are shown in Supplementary Table S9. The 1st strand cDNA synthesized was normalized based on the expression of the housekeeping gene *Solanum lycopersicum* elongation factor 1a (*SlEF1a*). The chosen housekeeping gene was stable among the samples processed and within the analysis conditions. In total, six to nine biological replicates of tomato RNA samples were analyzed with three technical replicates for each gene (Supplementary Table S10). The relative gene expression level ($2\text{-}\Delta\Delta C_t$) was calculated on Microsoft Excel according to Pfaffl (2001).

Statistical analysis

For the Y-tube olfactometer assays, the null hypothesis that the females of *M. pygmaeus* showed no preference for either arm of the olfactometer was tested using χ^2 test in R (Team 2013). Differences in the peak intensities measured for the targeted volatile compounds and the expression levels of the selected genes were analyzed in R (Team 2013). Two-way analysis of variance (ANOVA) was used to determine the effect of the root microbes used to inoculate the roots of the plants, the effect of herbivory and the combined effect of the two afore-mentioned factors. Tukey multiple comparisons of means followed ANOVA to identify significant differences among the treatments. Prior to two-way ANOVA, all the data were log-transformed. Levene's test was used to test the homogeneity of variance across groups, and Shapiro test was used to check for the normal distribution of residuals. When the assumptions for two-way ANOVA were not met, one-way ANOVA was conducted only for the samples collected from herbivore-infested plants, followed by Tukey post hoc tests. Prior to one-way ANOVA, all the data were log-transformed. In the cases, where the assumptions for one-way ANOVA were not met, Kruskal Wallis test was performed for the samples collected from herbivore-infested plants, followed by Dunn's multiple comparisons test. Boxplots were plotted in R using the package ggplot2 (Wickham, 2016). Principal Component Analysis (PCA) was conducted in R (Team 2013) to explain the variation in the volatile peak intensity data.

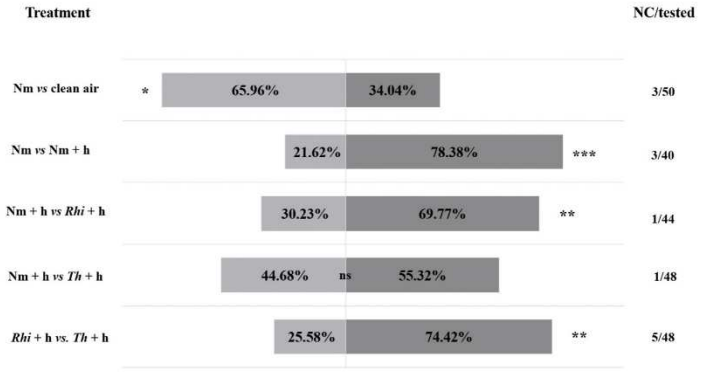

FIGURE 1
Responses of *Macrolophus pygmaeus* female predators to the volatiles emitted by non-inoculated (Nm), non-inoculated + *S. exigua* (Nm + h), *Rhizophagus irregularis*-inoculated + *S. exigua* (*Rhi* + h), *Trichoderma harzianum*-inoculated + *S. exigua* (*Th* + h) plants or clean air (air). At the sides of each bar, are shown the percentages of the predator females that moved towards the corresponding odor source based on the comparison indicated on the left side of the bars. The no choice/tested column on the right of each bar indicates the number of female predators that made no choice over the total number of tested predators (44–50 predators depending on the treatment combination were tested). In total, 13 out of 230 predators failed to make a choice. Asterisks indicate significant differences after performing a χ^2 test: ns ($p > 0.05$), * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$).

Results

Macrolophus pygmaeus choice

By using a Y-tube olfactometer, we investigated whether the inoculation of tomato roots with beneficial microbes could affect the attraction of the omnivorous predator *M. pygmaeus* towards *S. exigua*-infested plants. Therefore, we set up a Y-tube olfactometer assay, where *M. pygmaeus* females were allowed to make a choice between the following options: 1) non-inoculated tomato plants versus clean air (Nm *vs.* air), 2) non-inoculated versus non-inoculated *S. exigua*-infested plants (Nm *vs.* Nm + h), 3) non-inoculated *S. exigua*-infested versus *R. irregularis*-inoculated *S. exigua*-infested plants (Nm + h *vs. Rhi* + h), 4) non-inoculated *S. exigua*-infested versus *T. harzianum*-inoculated *S. exigua*-infested plants (Nm + h *vs. Th* + h) and 5) *R. irregularis*-inoculated *S. exigua*-infested versus *T. harzianum*-inoculated *S. exigua*-infested plants (*Rhi* + h *vs. Th* + h).

Our results showed that *M. pygmaeus* females preferred Nm tomato plants to clean air (Figure 1, χ^2 (1, n = 47) = 4.79; p = 0.03). In addition, the females were strongly attracted by the volatiles emitted by Nm + h infested plants over the Nm ones (Figure 1, χ^2 (1, n = 37) = 11.92; p = 0.0006). The comparison between Nm + h and *Rhi* + h plants, showed a stronger preference to the *Rhi* + h ones (Figure 1, χ^2 (1, n = 43) = 6.72; p = 0.001). The volatiles emitted by *Th* + h plants, attracted more female predators than the volatiles emitted by Nm + h plants. However, the difference between the two was not statistically significant (Figure 1, χ^2 (1, n = 47) = 0.53; p = 0.47). The comparison between *Rhi* and *Th* plants both under herbivory showed that the female predators were stronger attracted by the *Th* + h over the *Rhi* + *h* plants (Figure 1, χ^2 (2, n = 43) = 10.3; p = 0.01).

Tomato volatiles

In order to investigate the compounds that might be responsible for affecting the choices of *M. pygmaeus* females recorded in the Y-tube olfactometer assays, we collected volatiles from non-inoculated and root-inoculated plants with and without *S. exigua* larvae infestation (Nm, Nm + h *Rhi*, *Rhi* + h, *Th*, *Th* + h). Targeted analysis of our volatile data resulted in a list of 18 compounds that were further analyzed. Among them, we (tentatively) identified methyl salicylate, compounds belonging to the distinct classes of C6 green leaf volatiles (GLVs), monoterpenes and sesquiterpenes.

Principal component analysis (PCA) revealed that the volatile emissions of herbivore-infested plants differed from the volatile emissions of non-herbivore-infested plants. As shown by PCA, the first two principal components explained 69.4% of combined variance and separated our samples between herbivore- and non-herbivore-infested (Figure 2). *Spodoptera exigua* herbivory upon the leaves of tomato is considered the factor driving the separation among our samples.

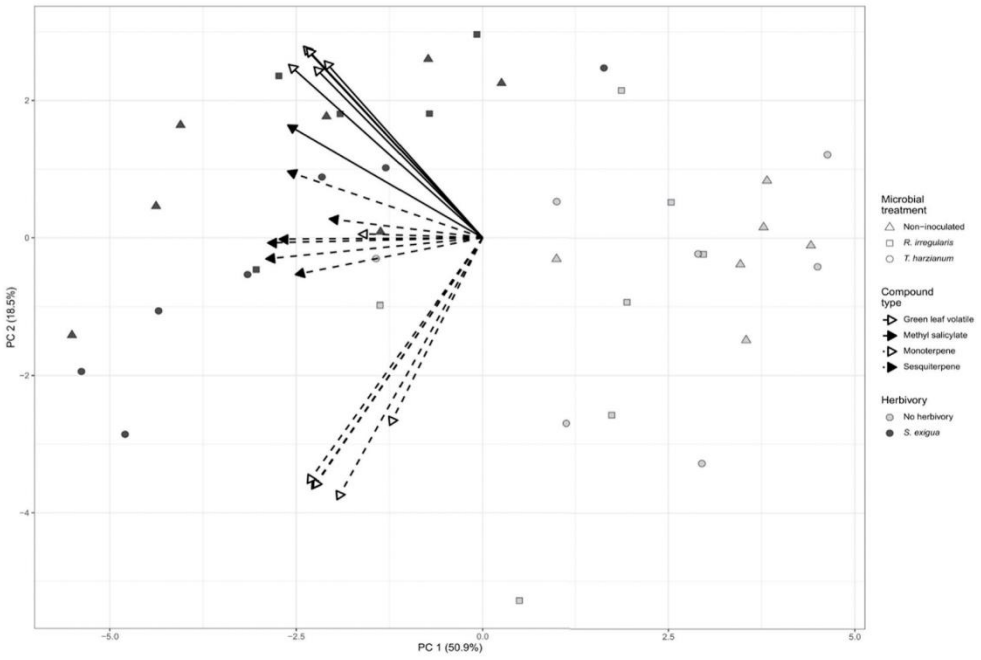

FIGURE 2
Principal component analysis (PCA) biplot describing the volatile emissions of non-inoculated (Nm), *Rhizophagus irregularis*-inoculated (*Rhi*), *Trichoderma harzianum*-inoculated (*Th*) tomato plants non-infested or infested by *Spodoptera exigua* larvae for 24 h. Principal component analysis (PCA) was performed for 18 selected volatile compounds. Symbols represent the biological replicates within each microbial treatment: Nm (triangle); *Rhi* (square) *Th* (circle). Filled symbols represent *S. exigua*-infested plants, whereas not filled symbols represent non-infested plants. The arrows point to the volatile organic compound classes that contributed to the separation of the samples based on our targeted analysis. Continuous line arrows without a filled head point to the green leaf volatiles; with a filled head point to methyl salicylate. Perforated line arrows without a filled head point to monoterpenes; with a filled head point to the sesquiterpenes.

Next, we investigated whether herbivory of *S. exigua* upon tomato leaves, tomato root inoculation with beneficial microbes and the interaction effect between the two aforementioned factors influenced the induction of the targeted volatile compounds. For the GLVs, *S. exigua* herbivory demonstrated a predominant effect on the emission of three compounds. In particular, this was observed for the green leaf volatile cis-3-hexenylacetate (p_h = 0.002, Table 1; Supplementary Table S2; Figure 3A), the compound identified as cis-3-hexenylbutyrate (p_h = 2.06e-05, Table 1; Supplementary Table S2; Figure 3B) and the compound tentatively identified as cis-3 hexenyl isovalerate (p_h = 0.0001, Table 1; Supplementary Table S2; Figure 3C). For the other two GLVs [unknown GLV 1 and the compound tentatively identified as 3-hexen-1-ol, propanoate, (*Z*)-] in our analysis, two-way ANOVA showed that none of the factors tested influenced the release of these compounds from tomato plants in response to larval infestation (Table 1; Supplementary Table S2; Supplementary Figure S1A,B, respectively). The interaction effect between herbivory and beneficial root microbes was not shown as significant for any of the five GLVs found. Similarly, root inoculation of our experimental plants with beneficial microbes prior to herbivory did not affect the synthesis and emission of any of the green leaf volatiles we focused on. Herbivory by *S. exigua* larvae significantly affected the emission of the volatile methyl salicylate (MeSA) (p_h = 9.2e-10, Table 1; Supplementary Table S2; Figure 3D) as well. Both the effect of root symbionts and the interaction effect between herbivory and the root symbionts used were not significant in shaping MeSA emissions.

In our volatile analysis, we also targeted six monoterpenes. The effect of root symbionts was marginally significant for the emission of the monoterpene tentatively identified as α-phellandrene (p_m = 0.08, Table 2; Supplementary Table S3; Supplementary Figure S2B) and the monoterpene tentatively

TABLE 1 Volatiles emitted by tomato plants according to their measured retention time and calculated Kovats retention index (RI).

Volatile	Calculated kovats RI	Two-way ANOVA		
		Microbe (m)	Interaction (h x m)	Herbivore (h)
cis-3-hexenyl acetate[a]	1318	0.101	0.484	0.002 (**)
unknown GLV1	1379	0.631	0.105	0.271
3 hexen-1-ol, propanoate (Z)-[b]	1384	0.672	NA	0.99
cis-3-hexenyl butyrate[a]	1462	0.349	0.551	2.06e-05 (***)
cis-3-hexenyl isovalerate[c]	1473	0.331	0.76	0.0001 (***)
methyl salicylate	1790	0.476	0.369	9.2e-10 (***)
unknown monoterpene 1	1141	0.127	0.368	0.144
limonene[a]	1207	0.186	0.071 (.)	0.023 (*)
β-phellandrene[d]	1219	0.189	0.106	0.036 (*)
trans-β-ocimene[a]	1254	0.574	0.349	0.025 (*)
unknown sesquiterpene 1	1595	0.273	0.8	0.88
caryophellene[a]	1621	0.818	0.071 (.)	4.72e-05 (***)
unknown sesquiterpene 2	1654	0.086 (.)	0.274	0.312
γ-muurolene[d]	1685	0.159	0.346	0.001 (**)
α-humulene[d]	1690	0.983	0.2	0.0002 (***)

[a]Authentic standard.
[b]Werkhoff et al. (1998).
[c]Kawakami et al. (1995).
[d]Lopes et al. (2004).
Compounds were identified by comparison to an authentic standard[a] or tentatively identified by comparison to RI values in the literature[b,c,d], when possible. In this table, the volatile compounds of which the *p*-values have been calculated with two-way ANOVA are presented with *Microbe* (m), *Herbivore* (h) and their interaction (m x h) as factors. Asterisks indicate significant differences after performing two-way ANOVA: * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

identified as α-terpinene (p_m = 0.06, Table 2; Supplementary Table S4; Supplementary Figure S2A). As the post hoc tests showed, there was a marginally significant difference in the emission of the monoterpene α-phellandrene between the *T. harzianum*- and *R. irregularis*-inoculated plants (p_{adj} = 0.08, Supplementary Table S3). Similarly, a significant difference was found between the non-inoculated and mycorrhizal plants under herbivory in the release of the monoterpene α-terpinene (p_{adj} = 0.05, Supplementary Table S4). On the other hand, *S. exigua* herbivory was the main factor driving the emission of the monoterpene identified as limonene (p_h = 0.02, Table 1; Supplementary Table S2; Figure 3E). Interestingly, for the same volatile compound, the interaction effect between herbivory and the root symbionts used was marginally significant ($p_{m\ x\ h}$ = 0.07, Table 1; Supplementary Table S2). Conversely, the emission of the monoterpene tentatively identified as β-phellandrene was significantly affected exclusively by *S. exigua* herbivory at p_h = 0.03 (Table 1; Supplementary Table S2; Figure 3F). Herbivory had the same significant effect on the emission of the monoterpene identified as trans-β-ocimene and p_h = 0.02, (Table 1; Supplementary Table S2; Supplementary Figure S1D).

Apart from monoterpenes, in our analysis, we also focused on six sesquiterpenes. Herbivory strongly affected the levels of caryophellene (p_h = 4.72e -05, Table 1; Supplementary Table S2; Figure 3G). In addition, a weak interactive effect between *S. exigua* herbivory and the root symbionts was found for the same compound ($p_{h\ x\ m}$ = 0.07, Table 1; Supplementary Table S2). Similarly, the beneficial microbes used to inoculate the plants were shown to marginally influence the emission levels of the unknown sesquiterpene 2 (p_m = 0.08, Table 1; Supplementary Table S2; Supplementary Figure S1F). Lastly, for the sesquiterpenes tentatively identified as γ-muurolene and α-humulene, larval herbivory was shown as the main factor influencing their emission with p_h = 0.001 and p_h = 0.0002, respectively (Table 1; Supplementary Table S2; Figure 3H,I, respectively).

Tomato indirect defense gene expression

In our gene expression analysis, we focused on selected genes, which are involved in the jasmonic acid, salicylic acid and terpenoid biosynthetic pathways. For the jasmonic acid pathway-related genes, we observed that the transcriptional levels of the lipoxygenases *LOXA* and *LOX* were not significantly affected by the root symbionts (one-way ANOVA, p_m = 0.58 (Supplementary Table S7; Supplementary Figure S3B) and p_m = 0.5 (Supplementary Table S7; Supplementary Figure S3A) for *LOXA* and *LOX*,

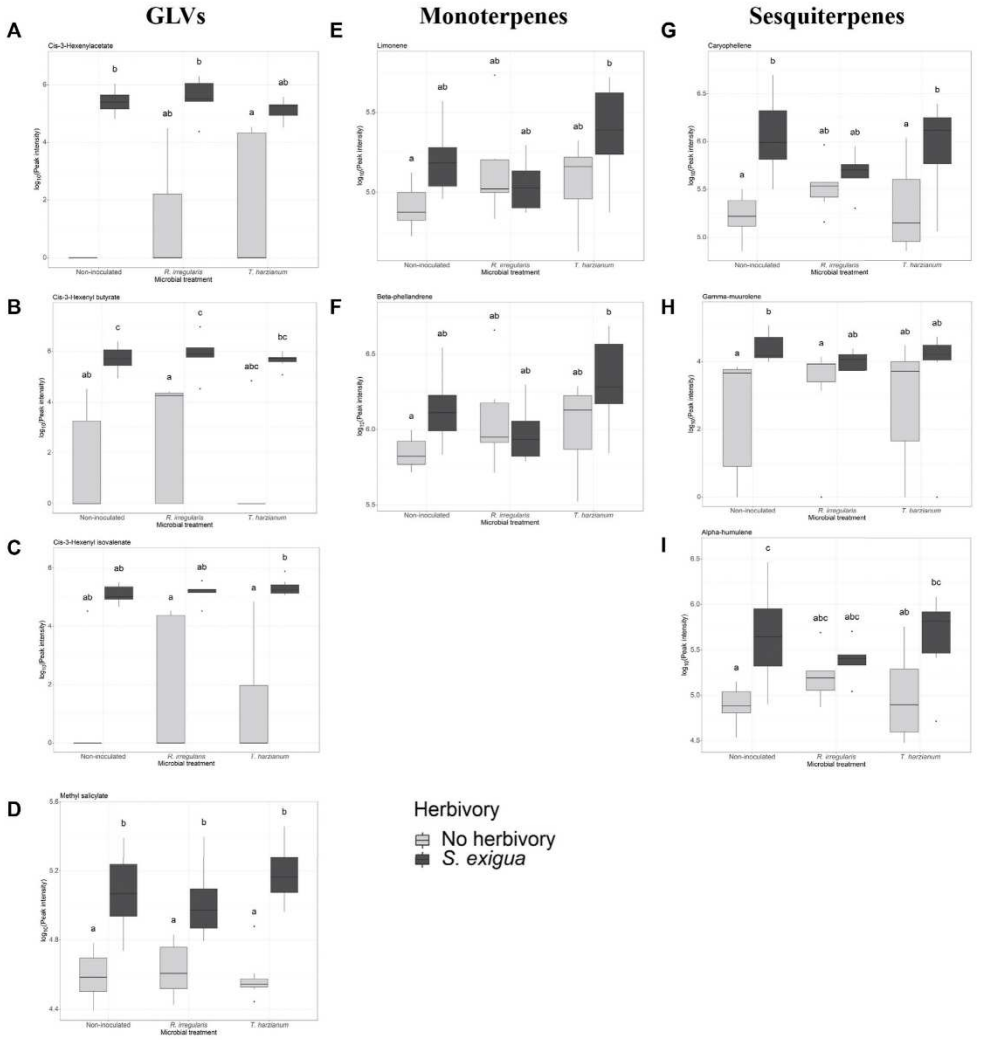

FIGURE 3
Selection of volatile compounds emitted by non-inoculated and root-inoculated tomato plants non-infested or infested by *Spodoptera exigua* larvae for 24 h. The compounds depicted belong to the class of green leaf volatiles **(A–C)**, methyl salicylate **(D)**, monoterpenes **(E,F)**, and sesquiterpenes **(G–I)**. The compounds shown were selected based on the effect of *S. exigua* herbivory on their emission. Treatments are: control plants symbolized as Non-inoculated, *Rhizophagus irregularis*-root inoculated plants symbolized as *R. irregularis* and *Trichoderma harzianum* root-inoculated plants symbolized as *T. harzianum*. Light gray-colored boxplots represent non-herbivore-infested plants and dark gray-colored boxplots represent *S. exigua*-infested plants. Significant differences between treatments are indicated by different letters after Tukey's *post hoc* tests after two-way ANOVA: $p < 0.05$.

respectively). Conversely, herbivory was the main factor driving the expression levels of the gene *allene oxide synthase 2* (*AOS2*; p_h = 2.389e-10 (Supplementary Table S5, S6; Figure 4A). However, neither the beneficial root microbes nor the interactive effect of herbivory and root symbionts significantly affected *AOS2* gene expression.

TABLE 2 Volatiles emitted by tomato plants according to their measured retention time and calculated Kovats retention index (RI).

Volatile compound	Calculated kovats RI	One-way ANOVA	Kruskal–Wallis test
		microbe (m)	microbe (m)
α-phellandrene[a]	1175	0.086 (.)	-
α-terpinene[a]	1187	-	0.065 (.)
unknown sesquiterpene 3	1707	-	0.858

[a]Lopes et al. (2004).

Compounds were identified by comparison to an authentic standard[a] or tentatively identified by comparison to RI values in the literature[b,c,d], when possible. In this table, the volatile compounds of which the *p*-values have been calculated with one-way ANOVA or Kruskal–Wallis test among herbivore-infested samples are presented with *Microbe* (m) as factor. Asterisks indicate significant differences after performing one-way ANOVA or Kruskal–Wallis test: * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

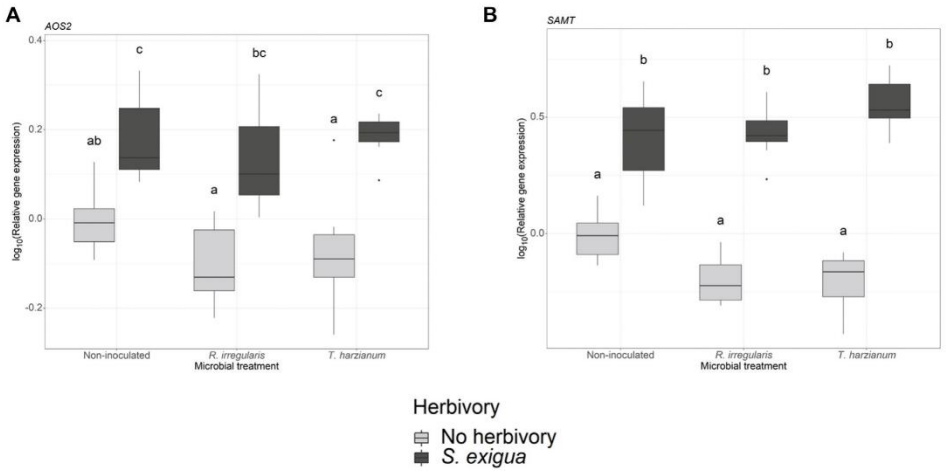

FIGURE 4
Transcript levels of two defense-related genes in non-inoculated and root-inoculated tomato plants non-infested or infested by *Spodoptera exigua* larvae for 24 h. Panel **(A)** depicts the transcript levels of the gene allene oxide synthase 2 (AOS2), and panel **(B)** depicts the transcript levels of the gene salicylic acid methyl transferase (SAMT). The genes depicted were selected based on the effect of *S. exigua* herbivory, or beneficial microbes on their transcript levels. Values are the average of the three technical replicates for each of six-seven biological replicates per treatment. Expression levels for the selected genes were normalized to the mean of tomato elongation factor 1a gene expression levels in each sample as reference. Light gray-colored boxplots represent non-herbivore-infested plants and dark gray-colored boxplots represent *S. exigua*-infested plants. Significant differences between treatments are indicated by different letters after Tukey's *post hoc* tests after two-way ANOVA: $p < 0.05$.

As a representative of terpenoid biosynthesis, we investigated the expression levels of the terpene synthase gene, *TPS5*. As it was shown, among the herbivore-infested samples, the root symbionts had no significant effect in determining the *TPS5* gene expression levels (p_m = 0.29, Supplementary Table S8; Supplementary Figure S3D).

We also measured the transcript levels of two genes involved in the salicylic acid (SA) biosynthetic pathway. At first, one-way ANOVA among the samples collected from larvae-infested plants, showed that arbuscular mycorrhiza and *T. harzianum* did not affect the expression of the gene phenylalanine ammonia-lyase (*PAL*) (p_m = 0.45, Supplementary Table S7; Supplementary Figure S3C). In the same pathway, we also measured the transcript levels of the gene salicylic acid methyl transferase (*SAMT*). Two-way ANOVA showed that herbivory strongly influenced the expression of *SAMT* (p_h < 2.2e-16, Supplementary Table S5, S6; Figure 4B). Interestingly, the interaction effect between *S. exigua* infestation and the root symbionts also played a significant role in modulating the expression levels of this gene ($p_{h \times m}$ = 0.004, Supplementary Table S5, S6).

Discussion

In our study, we found that tomato plants damaged by *Spodoptera exigua* caterpillars were highly attractive to *Macrolophus pygmaeus* females. Among herbivore infested plants, plants inoculated with beneficial microbes were more attractive, in particularly those infested with the mycorrhizal fungus *Rhizophagus*. However, when given the choice among the two, *M. pygmaeus* preferred herbivore- induced plants inoculated with *Trichoderma*. Comparative volatile and gene transcription analysis of herbivore-induced plants with and without beneficial microbes, however, did not yield a clear mechanistic basis for the observed choice patterns. The only exception to this might be the interactive effect of caterpillar and beneficial microbes on *SAMT*, the gene responsible for MeSA production. MeSA is well-known as a volatile cue attracting predators, including *Macrolophus* (Silva et al., 2021).

However, we found a strong effect of herbivore feeding on predator response, which we could clearly link to volatile emissions. Among non-inoculated plants, female predators were stronger attracted by the herbivore-damaged over undamaged plants. As shown in our volatile analysis, *S. exigua* larval herbivory resulted in the enhanced emission of a blend of volatiles including GLVs, such as cis-3-hexenyl acetate, methyl salicylate, and several terpenoids, such as β-phellandrene, trans-β-ocimene, caryophellene and α-humulene. Therefore, these volatile compounds may have affected the attraction of the female predators towards herbivore-damaged plants. In accordance with our findings, studies have shown that the GLVs (*Z*)-3-hexen-1-ol and (*Z*)-hexenyl acetate, and the volatile methyl salicylate determine the attraction of several parasitoid and predator species including *M. pygmaeus* (James, 2003a; b;Moayeri et al., 2007; Uefune et al., 2011; Salamanca et al., 2017; Silva et al., 2021). Moreover, the terpenoids β-phellandrene, (*E*)-ocimene, (*Z*)-ocimene, β-caryophellene and α-humulene have also been shown to attract the predator *M. pygmaeus* (De Backer et al., 2015). The strong effect of *S. exigua* herbivory was also depicted by the upregulation of *AOS2* and *SAMT,* genes involved in the oxylipin and phenylpropanoid pathways, respectively. These two pathways play a major role in HIPV biosynthesis. Notably, in the case of *SAMT,* an interaction effect between herbivory and beneficial microbes was also detected, underlining the ability of root symbionts to enhance indirect defense in response to herbivory and increase the attraction of natural enemies (Schausberger et al., 2012; Battaglia et al., 2013).

Among herbivore-infested plants, female predators showed significantly higher attraction towards *R. irregularis*-inoculated plants compared to the non-inoculated ones. Several studies have shown that beneficial root microbes, such as mycorrhizal fungi, are capable of modulating plant defense responses through the activation of induced systemic resistance or priming (Pineda et al., 2010; Martínez-Medina et al., 2017; Rasmann et al., 2017; Shikano et al., 2017). Our volatile analysis showed that mycorrhization only weakly affected the emission of a monoterpene, tentatively identified as α-terpinene. Notably, α-terpinene has been reported as one of the most influencing compounds on determining the attraction of *M. pygmaeus* predators towards *T. absoluta*-infested tomato plants (De Backer et al., 2015). The study of Prieto et al. (2017) reported improved foraging behavior and life history traits of *M. pygmaeus* on tomato plants root-inoculated with the isolate BEG 72 of *R. irregularis*. In particular, the authors showed that mycorrhizal plants attracted both female and male predators more strongly, were highly preferred for oviposition by reproductive females, and subsequently hosted a higher number of newborn nymphs (Prieto et al., 2017). Even if the object of the aforementioned study was not the investigation of the volatiles emitted by mycorrhizal and non-mycorrhizal tomato plants, the authors hypothesized that quantitative and/or qualitative changes in the blend of VOCs released might have mediated the higher attraction of the predator towards the leaves of mycorrhizal plants. We thus hypothesize that the volatile α-terpinene might have played an important role in the stronger attraction of *M. pygmaeus* towards mycorrhizal herbivore-infested plants in our study. Our finding combined with the results of Prieto et al. (2017) indicates that root colonization by *R. irregularis* could facilitate the establishment of the *Macrolophus* colonies in tomato crops in fields or greenhouses, provided there is abundance of prey, thus resulting in an effective and more sustainable way to protect the crops against herbivores.

Our results showed that root inoculation with beneficial fungi did not alter the expression levels of the *TPS5* gene in response to caterpillar herbivory. In our study, we investigated the expression levels of the gene coding for *TPS5*, which has been determined as a linalool synthase (Cao et al., 2014). The synthesis of α-terpinene in tomato is catalyzed by the enzymes *TPS9* and *TPS20* (Zhou and Pichersky, 2020), which might be the reason we did not observe any effect of mycorrhization on the expression levels of the terpene synthase gene chosen to be tested.

Several studies have reported significantly higher attraction of parasitoids and predators towards *Trichoderma*-inoculated tomato plants (Battaglia et al., 2013; Coppola et al., 2017). However, in our Y-tube experiments, the female predators did not prefer *T. harzianum*-inoculated to non-inoculated plants. A reason for this difference might be the different fungal strains, insect herbivores and/or tomato plant cultivars used in various studies.

Considering the above, it was all the more interesting that herbivore-induced plants with *T. harzianum* were significantly more attractive to predator females than the mycorrhizal plants. Our volatile analysis showed there was a marginally significant difference between *T. harzianum*-inoculated and mycorrhizal plants regarding the emission of a monoterpene, tentatively identified as α-phellandrene. This is in line with other studies, reporting that *Trichoderma spp*. can alter the concentration of

terpenoids emitted by host plants in response to herbivory (Battaglia et al., 2013; Contreras-Cornejo et al., 2018a; Contreras-Cornejo et al., 2018b). Alpha-phellandrene was annotated among the volatile compounds emitted by *T. longibrachiatum* MK1-inoculated tomato plants, even if not significant (Battaglia et al., 2013). The same plants exhibited promoted plant development and were significantly more attractive for *M. pygmaeus* compared to the non-colonized ones (Battaglia et al., 2013). The difference observed in the degree of significance of α-phellandrene between our study and the study of Battaglia et al. (2013) might be attributed to the difference between the *Trichoderma* strains used. We thus hypothesize that α-phellandrene might be involved in the mechanism used by *Trichoderma* strains to induce indirect defense responses against herbivores in tomato plants. In this frame, sustainable crop protection strategies that employ *M. pygmaeus* zoophytophagous predators and *Trichoderma* fungal strains could work synergistically in protecting tomato crops against herbivores.

To our knowledge, this is the first study so far, to compare the responses of female predators towards mycorrhizal and *Trichoderma*-inoculated tomato plants under herbivory. Arbuscular mycorrhizal fungi and *Trichoderma* species use different mechanisms of colonization and induction of biochemical, physiological and molecular responses on the host plans (Strack et al., 2003; Smith and Smith, 2011; Hermosa et al., 2012). Therefore, we hypothesize that the presence of *T. harzianum* on tomato roots might led to slightly increased emission of α-phellandrene compared to the mycorrhizal plants. This difference in the volatile blend of *T. harzianum* plants might subsequently influenced the responses of *M. pygmaeus* female predators. Overall, both α-phellandrene and α-terpinene showed higher emissions in the microbe-inoculated plants, thus we assume that the two compounds may have influenced the attraction of the predator. *Macrolophus pygmaeus* predators are broadly released in greenhouses to protect tomato crops against herbivores (Castañé et al., 2004; Urbaneja et al., 2012). Therefore, choice experiments to compare the responses of female predators to the volatile compounds α-phellandrene and α-terpinene are required to elucidate which of the two compounds might be more effective.

Conclusion

Collectively, our results show that, despite the dominant effect of herbivory on the synthesis and emission of volatiles; beneficial microbes also show potential of altering the biosynthesis and release of these compounds. Our findings combined with the results of other studies in the field pinpoint the role that root symbionts could play in the application of integrated pest management practices and sustainable agriculture. To this direction, more studies investigating the ability of different beneficial microbes to modulate indirect defenses in response to herbivory through the attraction of natural enemies of the insect herbivores are required.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.zenodo.org/, https://doi.org/10.5281/zenodo.6698584.

Author contributions

NvD, AW, DP, and DC conceived the idea and designed the study. DP and DC conducted the experiments and collected the data. DP and DC analyzed and interpreted the data; assisted by AW in the case of the volatile collection data. DP and DC wrote the first draft of the manuscript. NvD, AM-M, and AW reviewed and edited the manuscript. NvD, AM-M, and AW contributed to funding acquisition. NvD and AW contributed to the study supervision. All authors have read and agreed to the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.1003746/full#supplementary-material

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General discussion

My research presented in this dissertation showed that the beneficial root microbes *R. irregularis* and *T. harzianum* mediate plant-insect interactions. Their presence in the roots of tomato was associated with important ecological effects on several levels. The two root mutualists caused a widespread re-arrangement of herbivore-induced plant responses at the plant transcriptome/metabolome level (Chapter 2) and in the emission of herbivore-induced plant volatiles (Chapter 3), as well. Remarkably, they negatively affected the performance of the specialist insect herbivore *M. sexta* (Chapter 1) and enhanced the attraction of a third trophic level insect, the predator *M. pygmaeus* (Chapter 3) towards *S. exigua*-infested tomato plants. The combination of insect performance bioassays with untargeted plant and insect metabolomics (Chapter 1); the integration of plant RNA-sequencing with untargeted plant metabolomics (Chapter 2); and the combination of olfactometer bioassays with targeted volatile and plant gene expression analyses (Chapter 3) were used to link the observed biological effects with changes in the plant defense phenotypes triggered by beneficial microbes. In conclusion, the current study provides evidence that the two beneficial microbes caused important effects over three trophic levels, namely the plant, its herbivores, and their natural enemies. We discuss the main results of this Doctoral thesis in the frame of (1) the impact of the mutualistic microbes on plant anti-herbivore response, including defense regulation and changes in the secondary metabolism of the plant; (2) the impact of the mutualistic microbes on the physiology of the insect herbivores; and (3) the impact of the mutualistic microbes on the third trophic level.

1. Impact of beneficial root fungi on plant anti-herbivore responses

Root mutualistic fungi are able to affect plant resistance against arthropod pests (Pineda et al., 2015; Pineda et al., 2010; Ramamoorthy et al., 2001), either through changes in plant vigor, or through changes in plant endogenous regulators, and ultimately plant defenses (Pieterse et al., 2014; Pineda et al., 2010; Pozo & Azcón-Aguilar, 2007; Van der Ent et al., 2009; Van Wees et al., 2008; Vannette & Hunter, 2009). Colonization by arbuscular mycorrhizal fungi (AMF) is reported to evoke stronger and faster defense responses in the host plant, in comparison with plants that are not colonized, leading to the expression of mycorrhizal-induced resistance [MIR; (Jung et al., 2012; Pozo & Azcón-Aguilar, 2007; Pozo et al., 2010)]. A number of studies have confirmed the major role of jasmonic acid (JA) in regulating mycorrhizal-induced resistance (He et al., 2017; Jung et al., 2012; Nair et al., 2015; Pozo* et al., 2009; Schoenherr et al., 2019; Song et al., 2013). By following an untargeted transcriptomic approach, such as RNA sequencing, in this study, I aimed to investigate novel mechanisms that might underlie MIR.

1.1 Induced resistance triggered by beneficial root fungi is concomitant with the regulation of the ethylene- and auxin-regulated pathways

Beneficial root microbes, such as AMF, plant growth-promoting fungi and rhizobacteria (PGPF and PGPR, respectively) can induce plant systemic immunity, called induced systemic resistance (ISR) (Pozo & Azcón-Aguilar, 2007; Van Loon et al., 1998). Several phytohormones have been shown to mediate microbe-induced resistance (Pieterse et al., 2009; Pieterse et al., 2014). Among them, JA holds a prominent role (Pieterse et al., 1996; Pozo et al., 2008; Van der Ent et al., 2009; Van Loon et al., 1998; Van Wees et al., 2008). Nevertheless, as more resistance-inducing agents are being characterized, the involvement of additional signaling pathways in the induction of plant resistance becomes evident. To this direction, untargeted approaches can prove helpful tools to unravel other phytohormones that might contribute to the regulation of microbe-induced resistance as well. As shown in Chapter 2, genes involved in ethylene signaling were differentially expressed in *T. harzianum*- and in *R. irregularis*-inoculated plants under herbivory. In particular, genes coding for isoforms of the ethylene synthesis enzyme 1-aminocyclopropane-1 carboxylate synthase (ACC synthase) and the APETALA2/ETHYLENE RESPONSIVE FACTOR (AP2/ERF) transcription factor (TF) were shown as differentially expressed in *Trichoderma*-plants after *M. sexta* herbivory. *Trichoderma* spp. are able to induce JA-/ET-regulated ISR, resulting in enhanced plant protection against biotic stressors (Segarra et al., 2009; Shoresh et al., 2005; Tucci et al., 2011). In accordance with these results, genes coding for several ERF TFs were also upregulated in tomato plants root-inoculated with *T. harzianum* T22 and *T. atroviride* P1 (Coppola, Cascone, et al., 2019; Coppola, Diretto, et al., 2019). Furthermore, ERF TFs are involved in innate plant immunity. For example, in *Arabidopsis*, AP2/ERF TFs are involved in JA-inducible gene expression, and are known as octadecanoid-responsive elements, which positively regulate the expression of JA- and ET-mediated defense-related genes (Pré et al., 2008). In analogy to *T. harzianum*-inoculated plants, the transcriptomic analysis also showed the upregulation of ethylene responsive factor (ERF) genes in the mycorrhizal plants under herbivory. It has been suggested that ethylene holds a regulatory role in mycorrhizal-induced resistance (López-Ráez et al., 2010; Pozo et al., 2010). Moreover, in several studies, ethylene-responsive TFs have been reported among the highly upregulated genes in AMF-plants, suggesting the regulatory role they may hold (Cervantes-Gámez et al., 2016; Fiorilli et al., 2009; Liu et al., 2007). Taken together, these results seem to indicate that the ethylene-regulated pathway was induced by AMF and *T. harzianum* resulting in the induction of plant defenses against *M. sexta* herbivory.

We also found that eight small auxin upregulated RNA (SAUR) genes were downregulated in the *T. harzianum*-inoculated plants after herbivory. Beneficial microbes are able to facilitate their establishment in the host plant through the manipulation of defense signaling pathways, such as the JA and salicylic acid (SA) pathways. At the same time, they are also capable of manipulating phytohormones, such as auxins, cytokinins (CKs), ET, abscisic acid (ABA), and gibberellins (GAs) (Van der Ent et al., 2009; Zamioudis & Pieterse, 2012). This hormonal modulation is involved not

only in the effect of beneficial microbes on plant defense, but also in plant growth and development (Guzmán-Guzmán et al., 2019). *Trichoderma* strains have been reported to produce auxin-related compounds (Contreras-Cornejo et al., 2009; Guzmán-Guzmán et al., 2019) upon plant colonization. The role of *Trichoderma*-synthesized auxin in root architecture has not been fully elucidated yet (Hoyos-Carvajal et al., 2009; Nieto-Jacobo et al., 2017). However, there is evidence that in *Arabidopsis* plants, *Trichoderma*-produced elicitors facilitate changes in root architecture and increase plant biomass through auxin signaling (Garnica-Vergara et al., 2016). Therefore, it is likely that in this study, inoculation of tomato roots with *T. harzianum* led to upregulation of the auxin signaling pathway and promotion of plant growth in the absence of herbivory. Nevertheless, it is possible that after *M. sexta* infestation, *T. harzianum*-inoculated plants shifted from growth to defense responses, hence resulting in the observed downregulation of *SAUR*.

1.2 Beneficial root fungi alter the levels of plant secondary metabolites and protease inhibitors in tomato plants

The metabolomic analysis conducted in Chapter 1 showed that root-inoculation with AMF and *T. harzianum* resulted in increased levels of secondary metabolites in tomato leaves. In particular, the levels of steroidal glycoalkaloids such as *α*-tomatine, *α*-dehydrotomatine and their isomers were elevated in the leaves of microbe-inoculated plants with and without herbivory. In addition, the transcriptomic analysis conducted in Chapter 2 revealed that a gene coding for anthocyanin 5-aromatic acyltransferase and genes coding for protease inhibitors were higher expressed in herbivore-infested *T. harzianum*-inoculated plants. To counter herbivore attack, plants produce secondary metabolites, such as phenolic compounds, terpenes, nitrogen- and sulphur-containing compounds; and proteins, such as protease inhibitors (PIs), which have toxic, repellent, and/or anti-nutritional effects on herbivores (Usha Rani & Jyothsna, 2010; War et al., 2012; War et al., 2018). Moreover, AMF and *Trichoderma* spp. have been reported to induce plant resistance against herbivores through alterations in plant secondary metabolism (Coppola, Diretto, et al., 2019; Rivero et al., 2021; Shrivastava et al., 2015).

As shown in Chapter 1, some secondary metabolites were over accumulated in the leaves tomato plants inoculated with beneficial fungi. Among these compounds, the alkaloid *α*-tomatine, which has a prominent role in tomato anti-herbivore defenses, was annotated. *Solanaceae* is a widespread family of species rich in alkaloids, including tropane alkaloids, glycoalkaloids (such as *α*-tomatine), pyrollizidine and indole alkaloids, which are naturally produced as a defense mechanism against insects (Jerzykiewicz, 2007). *Solanaceae* alkaloids exert both lethal and sub-lethal effects, and their toxicity is manifested at all levels of biological organization (Chowański et al., 2016). Alkaloids can affect nerve transmission in insects, disturbing the cell membrane and cytoskeletal structure, causing the collapse and leakage of cells (Mbata & Payton, 2013). Several

studies have reported the effects of pure *Solanaceae*-produced secondary metabolites on herbivorous insects. For instance, feeding of *M. sexta* larvae on artificial diet containing *α*-tomatine resulted in inhibition of larval growth (Weissenberg et al., 1998), and *S. exigua* larvae that were fed upon diet containing nicotine, exhibited increased lethality and decreased body mass (Kumar et al., 2014). Similarly, rearing of *Myzus persicae* aphids on artificial diets containing the alkaloids *α*-solanine and *α*-chaconine resulted in reduced feeding and fecundity of adults as well as reduced weight and increased mortality of nymphs (Fragoyiannis et al., 1998).

The transcriptomic analysis conducted in Chapter 2 revealed that *M. sexta* herbivory resulted in the upregulation of processes related to stress responses, including polyamine, secondary and amino acid metabolism. Interestingly, stronger shifts were observed in the *T. harzianum* root-inoculated plants, including the increased expression of a gene coding for anthocyanin 5-aromatic acyltransferase and three types of protease inhibitors Ⅰ and Ⅱ.

The anthocyanin 5-aromatic acyltransferase is involved in the synthesis of anthocyanins, which are synthesized via the flavonoid branch of the phenylpropanoid pathway. Anthocyanins protect plants against various abiotic and biotic stress factors (Cao et al., 2016; Chalker-Scott, 1999; Ren et al., 2014; Shafiq & Singh, 2018) and their biosynthetic pathway has been extensively studied in several plant species (Koes et al., 2005; Lepiniec et al., 2006; Quattrocchio et al., 1993). Flavonoids show insecticidal effects and help plants to ward off phytophagous insects (Wang et al., 2014; Züst & Agrawal, 2017). For example, Kariyat et al. (2019) showed that flavonoids present in wild type sorghum (*Sorghum bicolor* (L.) Moench family: *Graminaceae*) caused significantly higher mortality and reduced the population growth of the corn leaf aphid (*Rhopalosiphum maidis* Finch). Furthermore, the flavonoid-rich pericarp extract of corn (*Zea mays*) negatively affected the growth, development, and adult fitness traits in *M. sexta* (Tayal, Somavat, Rodriguez, Martinez, et al., 2020; Tayal, Somavat, Rodriguez, Thomas, et al., 2020). It is thus possible that root-inoculation with *T. harzianum* resulted in the enhancement of tomato plant defenses through the upregulation of the gene coding for the anthocyanin 5-aromatic acyltransferase. However, the expression levels of the gene phenylalanine ammonia lyase (*PAL*), which belongs to the early stage biosynthetic genes of the phenylpropanoid biosynthetic pathway, were unaffected by *T. harzianum* root inoculation, as shown in Chapter 3. The contradiction between these two results might be due to the difference between the lepidopteran caterpillars used in Chapters 1 and 3. *Manduca sexta* (used in Chapter 1) and *Spodoptera exigua* (used in Chapter 3) are both chewing herbivores, and hypothetically induce similar defense pathways in the host plant. Nevertheless, they differ in their degree of specialization and produce different elicitor molecules that are then perceived by the host plant and can differently influence its defense responses.

In this frame, the study of Bosch et al. (2014) reported that tomato plants responded differently to *S. exigua* and *M. sexta* feeding, suggesting that insect-derived molecules present in the oral secretions (OS) of the two herbivores are likely responsible for this dissimilarity. As they observed, the application of native *S. exigua* OS on wounded tomato leaves resulted in

substantial increase in the activity of polyphenol oxidase (PPO), which is a reliable marker of tomato defense response and insect performance (Constabel et al., 1995; Felton et al., 1989). On the contrary, application of *M. sexta* native OS on wounded tomato leaves caused a modest induction of foliar PPO activity. To get a deeper insight into this effect, the authors focused on the OS produced by each insect species. After denaturation of the OS of both herbivores by heat treatment, no difference was observed between *S. exigua* and *M. sexta* PPO-inducing activity. The authors hypothesized that the induction of PPO activity in tomato is mediated by a heat-labile, likely proteinaceous constituent that is present in *S. exigua* and not in *M. sexta* OS (Bosch et al., 2014). Providing that the fatty acid amino acid conjugates (FACs) that are known to differ in composition in the OS of the two insect species (Diezel et al., 2009) are heat stable (Engelberth et al., 2007; Roda et al., 2004), they were unlikely to be responsible for the marked difference. Thus, the authors proposed the enzyme glucose oxidase (GOX) as an obvious candidate.

Besides secondary metabolites, protease inhibitors (PIs) also have a prominent role in plant defenses against herbivores, as they cover one of the most abundant classes of defensive proteins in plants (Hanley et al., 2007; War et al., 2012). PIs bind to the digestive enzymes in the insect gut and inhibit their activity, thereby reducing protein digestion, resulting in shortage of amino acids, and slow development/starvation of insects (Azzouz et al., 2005; Haq et al., 2004; Santamaria et al., 2012; Zhu-Salzman & Zeng, 2015). Here, a higher upregulation of genes coding for three types of protease inhibitors (PIs) Ⅰ and Ⅱ was observed in the *T. harzianum*-colonized plants in response to herbivory. Similar to the results here, tomato plants root-inoculated with *T. atroviride* strain P1 exhibited enhanced levels of genes coding for protease inhibitors, and this effect was associated with the reduced development and survival of *S. littoralis* herbivores (Coppola, Cascone, et al., 2019).

Interestingly, a significant interactive effect between the secondary metabolite nicotine and the trypsin proteinase inhibitor (TPI) has been reported for wild tobacco, *Nicotiana attenuata*, which is the primary host plant of *M. sexta* (Steppuhn & Baldwin, 2007). In this study, the authors showed that nicotine by itself strongly affected *S. exigua* larval mass, whereas TPI alone was ineffective. However, the interaction between nicotine and TPI resulted in plants that were more resistant to herbivory. Therefore, the authors concluded that the resistance effect of TPI depends on the production of nicotine in *N. attenuata* plants. Although my study system included *M. sexta* insects feeding on another solanaceous species, *S. lycopersicum*, an interactive effect between *α*-tomatine and tomato protease inhibitors might have also contributed to the diminished performance of *M. sexta* larvae fed on *T. harzianum*-inoculated tomato plants.

1.3 Beneficial root fungi alter plant stress response

The transcriptomic analysis conducted in Chapter 2 revealed that for the *R. irregularis*-colonized plants, neither genes involved in the synthesis of secondary metabolites, nor genes coding for

protease inhibitors were upregulated in response to herbivory. However, two peroxidases, a gene regulating Radical-Induced Cell Death 1 (*RCD1*), and an early nodulin-like gene were exclusively upregulated in these plants. Plant peroxidases carry out various functions in a broad range of physiological processes during plant growth and development, wound healing, reactive oxygen species (ROS) removal, and defense response against pathogen or insect attack (Bindschedler et al., 2006; Cosio & Dunand, 2009; Daudi et al., 2012; Passardi et al., 2005). The finding about peroxidases in this study is in line with other studies, where mycorrhiza-colonized plants showed increased peroxidase activity, which resulted in increased plant resistance (Bastías et al., 2018; Li et al., 2019). ROS formation in plants under biotic and abiotic stress can also result in radical-induced cell death. For instance, in *A. thaliana* plants, the RCD1 protein has been associated to hormonal regulation of several stress-responsive genes (Ahlfors et al., 2004). In conclusion, it seems that mycorrhiza affect peroxidases and ROS detoxification in tomato plants, leading to enhanced resistance against insect herbivores.

2. Impact of beneficial root fungi on herbivore physiology

Plant secondary metabolites are able to repel insect herbivores or deter their feeding. Furthermore, they can cause direct toxic symptoms on herbivores, leading to inhibition of their development that can subsequently result in insect death (Divekar et al., 2022). However, the effect of mycorrhizal-induced resistance and *Trichoderma*-induced resistance on insect herbivore physiology have not been thoroughly explained yet. Therefore, in this study, I investigated the metabolites accumulated in the gut and fat body tissues of *M. sexta* insects reared on mycorrhiza- and *T. harzianum*-inoculated tomato plants, and the phenotypes of the insects, aiming to gain better insights into the impact of AMF and *T. harzianum* on insect herbivore performance.

2.1. Root colonization by beneficial fungi impairs *M. sexta* physiology and performance towards the insect life cycle

The majority of *M. sexta* larvae reared on *T. harzianum*-colonized tomato plants developed into abnormal pupae and exhibited higher mortality rates; whereas most of the larvae fed upon mycorrhizal tomato plants developed into moths with anomalous morphology that did not emerge. Several studies have reported the negative effect of *Trichoderma* strains on insect herbivores, in terms of growth and development (Alınç et al., 2021; Berini et al., 2016; Coppola, Cascone, et al., 2019), mortality (Contreras-Cornejo, Macías-Rodríguez, et al., 2018; Coppola, Cascone, et al., 2019; Coppola, Diretto, et al., 2019), and population growth (Pappas et al., 2021). In parallel, the effect of mycorrhization on herbivorous insects depends on the degree of specialization of the herbivore and its feeding guild. It can be summarized that mycorrhization

negatively affects generalist leaf chewers, while having a positive or neutral effect on phloem feeders and specialist chewers (Fontana et al., 2009; Gange et al., 2002; Gehring & Bennett, 2009; Goverde et al., 2000; Hartley & Gange, 2009; Koricheva et al., 2009; Pineda et al., 2010; Rivero et al., 2021; Vicari et al., 2002). In the present study, the metamorphosis of the specialist *M. sexta* was negatively affected when fed either on mycorrhizal or on *T. harzianum*-inoculated plants.

The analysis of the metabolites accumulated in the insect's tissues showed that piperidine was significantly increased in the guts of *M. sexta* larvae reared on *R. irregularis*-inoculated plants. Piperidine-containing alkaloids derive from the amino acid *L*-lysine. Piperidine-alkaloids can cause an acute toxic response to adult livestock animals by causing musculoskeletal deformities (Matsuura & Fett-Neto, 2015). Green et al. (2012) further stated that piperidine-containing alkaloids are responsible for teratogenic effects on mammals; and in humans, the accidental consumption of these compounds can induce toxicity symptoms (Hotti & Rischer, 2017).

Other metabolites found in *M. sexta* gut and fat bodies might have also contributed to the impaired insect metamorphosis observed in this study. Among these, carnitine-derived metabolites were annotated in the gut and fat body tissues of the larvae reared on microbe-inoculated plants. Furthermore, the metabolite annotated as octanoic acid was found in higher accumulation in the fat bodies of *T. harzianum*-reared larvae. Fat is always a major component of insect fat bodies (Arrese & Soulages, 2010). When the organism needs energy, stored fatty acids (FAs) are metabolized by hydrolysis (lipolysis) through catalysis by lipases. In turn, FAs can be used for the production of adenosine triphosphate (ATP) through β-oxidation (Van der Horst & Rodenburg, 2010). In addition, long-chain FAs must be conjugated to carnitine for effective transport across the mitochondrial membrane. In a recent study, changes in the abundance of acyl-carnitines were linked to changes in β-oxidation in *M. sexta* insects (Wone et al., 2018). Apart from being a source of energy, fatty acids have important physiological roles as components of cell membranes, cuticular lipids, waxes, as well as precursors in the synthesis of lipid regulators and hormones (Canavoso et al., 2001). Therefore, it is hypothesized that changes in the abundance of FAs in the gut and fat bodies of *M. sexta* larvae observed in this experiment might have affected either the β-oxidation process or other processes, in which FAs play a significant role, resulting thus in the impairment of *M. sexta* metamorphosis.

The levels of the metabolite annotated as benzamide were shown higher in the fat body tissues of the *T. harzianum*-fed larvae as well. Benzamides are simple derivatives of benzoic acid (Fellah et al., 2020) and have been widely used as pesticides. For instance, the recently discovered and commercialized benzamide-containing pesticide, broflanidine, is highly effective against various pests, including insect species in the orders of Lepidoptera, Thysanoptera and Coleoptera (Katsuta et al., 2019). Moreover, Mathé-Allainmat et al. (2012) identified the quiniclidine benzamide compound LMA10203, which acts as a partial agonist of the insect nicotinic acetylcholine receptor (nAChR) subtypes. Nicotinic acetylcholine receptors play an important role in the insect central nervous system because they are involved in learning and memory processes (Gauthier, 2010; Gauthier et al., 2006), and are specific targets of neonicotinoid

insecticides (Millar & Denholm, 2007; Tomizawa & Casida, 2003). Thus, it seems that the higher levels of benzamide annotated in the fat bodies of *T. harzianum*-fed larvae may have played a role in impeding *M. sexta* metamorphosis.

Root colonization by mutualistic microbes also altered the pattern of fatty acid amides (FAAs) in the gut and fat body tissues of *M. sexta* that were reared on these plants. This effect might also influence the elicitors of *M. sexta* larvae. Elicitors are referred to insect-derived compounds that activate plant defense pathways (Felton & Tumlinson, 2008). They are commonly found in insect OS and are able to upregulate defense genes and phytohormones and induce the synthesis of specific plant volatiles and secondary metabolites (Helms et al., 2014; Helms et al., 2013). However, insect elicitor compounds can also serve beneficial roles for the insect itself. For instance, a type of elicitors, the FACs enhance nitrogen metabolism for many caterpillar species (Helms et al., 2014; Helms et al., 2013; Helms et al., 2017; Mori & Yoshinaga, 2011; Yoshinaga et al., 2008). Therefore, changes occurring in the pattern of FAAs profile of *M. sexta* larvae reared on microbe-inoculated plants might subsequently hampered nitrogen assimilation for the herbivores, and this may explain the negative effects observed in their metamorphosis.

2.2 Beneficial root fungi possibly reduce the levels of glutathione in tomato plants

It was found that the levels of the metabolite annotated as glutathione were reduced in the guts of larvae fed on microbe-inoculated plants. Glutathione S-transferases (GSTs) are detoxification-related enzymes that belong to a multi-gene family (Eaton & Bammler, 1999). GSTs are involved in Phase Ⅱ detoxification of a wide variety of plant defensive chemicals and act by catalyzing the conjugation of glutathione to xenobiotics (Eaton & Bammler, 1999). Therefore, GSTs play a major role in the protection of specialized insect herbivores against plant secondary metabolites. Thus, the decreased levels of the metabolite annotated as glutathione in the guts of larvae reared on microbe-inoculated plants might be linked to the observed aberrant metamorphosis of the herbivores fed upon these plants.

2.3 Linking the insect and plant metabolomes

The analyses of plant and insect metabolome performed in Chapter 1 revealed that the abundance of the steroidal glycoalkaloid *α*-tomatine was higher not only in the leaves of microbe-inoculated plants, but also in the gut and fat body tissues of *M. sexta* larvae reared upon tomato plants root-inoculated with mutualistic microbes. In general, *M. sexta* is highly tolerant and adapted to detoxifying defensive chemicals (i.e., tomatine and nicotine) contained in its solanaceous host plants, compared to generalist insects feeding on the same plant species

(Glendinning, 2002; Wink & Theile, 2002). However, feeding of *M. sexta* larvae on artificial diet containing low and average levels of a combination of major tomato defensive metabolites, including also tomatine, resulted in reduced food consumption, extended the developmental time, and lowered the final weight of herbivores (Osier et al., 1996). In addition, a study examining the effect of differing levels of nicotine on *M. sexta* development, showed that the increase in nicotine concentrations contained in the artificial diet offered to *M. sexta* larvae was correlated with an almost linear decrease of their pupal mass (Harvey et al., 2007). Therefore, the results of the present study indicate that despite its high degree of specialization, *M. sexta* can be negatively affected by the specialized secondary metabolites produced by its host plants.

Based on the transcriptomic analysis of Chapter 2, genes involved in secondary metabolism (anthocyanin synthesis-related and PI-related) were higher upregulated in *T. harzianum*-inoculated tomato plants. Feeding of *M. sexta* larvae upon these plants would possibly cause oxidative stress effects on the herbivores. Consequently, the levels of metabolites participating in the detoxification process of plant defensive metabolites, such as glutathione, would be increased. However, in order to draw solid conclusions about the potential effects of plant defensive metabolites on *M. sexta* performance and life cycle are required: (1) additional experiments including the implementation of selected tomato defensive metabolites (i.e., *α*-tomatine, anthocyanins) in the diet of *M. sexta* larvae, and/or (2) experiments using tomato lines impaired in the synthesis of specific secondary metabolites.

3. Impact of beneficial root fungi on plant interaction with the third trophic level

Root mutualistic microbes are able to influence indirect defenses of plants and the recruitment of natural enemies of herbivorous pests (Babikova et al., 2013; Battaglia et al., 2013; Guerrieri et al., 2004; Katayama et al., 2011). Therefore, in this study, I aimed to understand the effect of *R. irregularis* and *T. harzianum* on the induction of tomato indirect defenses against the herbivore *S. exigua* and on the attraction of the predator *M. pygmaeus*.

3.1 Beneficial root fungi affect the profile of herbivore-induced plant volatiles and the attraction of natural enemies

I investigated the impact of *R. irregularis* and *T. harzianum* on indirect defenses of tomato plants in response to herbivory. With this aim, I analyzed the blend of volatiles emitted by microbe-inoculated plants under herbivory and measured the attraction of the zoophytophagous predator *M. pygmaeus* towards herbivore-infested plants. The volatile compound analysis conducted in Chapter 3 indicated that herbivory by caterpillars of *S. exigua* strongly enhanced the emission of

herbivore-induced plant volatiles, in particular green leaf volatiles (GLVs), methyl salicylate (MeSA) and terpenoids (mono- and sesquiterpenes). Root colonization by mycorrhiza slightly affected the release of a monoterpene tentatively identified as *α*-terpinene. In parallel, the volatile tentatively identified as *α*-phellandrene was found enhanced, albeit marginally significantly so, in *T. harzianum*-inoculated plants. The interaction effect between the herbivorous insect *S. exigua* and the two root symbionts significantly affected the expression levels of the gene coding for salicylic acid methyltransferase (*SAMT*). This enzyme catalyzes the production of the phenylpropanoid compound MeSA from SA. However, the emission of MeSA was exclusively affected by herbivory, indicating the dominant effect of herbivory in shaping tomato's volatile synthesis.

The olfactometer assays showed that among *S. exigua*-infested plants, the root-inoculated plants were more attractive to *M. pygmaeus*. Especially, in the case of mycorrhizal plants, we found a stronger attraction of the female predators compared to the non-inoculated plants. This result may be explained by the slightly enhanced emission of the volatile tentatively identified as *α*-terpinene from mycorrhizal plants. Alpha-terpinene has been reported to significantly affect the attraction of *M. pygmaeus* predators toward herbivore-infested tomato plants (De Backer et al., 2015). Interestingly, when comparing between microbe-inoculated plants, the predators preferred the *T. harzianum*-inoculated instead of the mycorrhiza-inoculated ones. This finding might be explained by the slighter enhanced emission of the volatile tentatively identified as *α*-phellandrene from T*. harzianum*-inoculated plants compared to the mycorrhizal ones. Alpha-phellandrene has been annotated in the blend of volatile compounds determining the preference of *M. pygmaeus* for tomato plants (Battaglia et al., 2013). Arbuscular mycorrhizal fungi and *Trichoderma* species use different mechanisms of colonization and induction of biochemical, physiological and molecular responses in their host plants (Hermosa et al., 2012; S. E. Smith & F. A. Smith, 2011). Thus, it is hypothesized that the presence of *T. harzianum* in the roots of herbivore-infested plants might resulted in slightly increased emission of *α*-phellandrene in comparison with the mycorrhizal plants. Such effect might subsequently influence the preference of *M. pygmaeus* predators. However, choice experiments comparing the responses of *M. pygmaeus* toward the volatile compounds *α*-phellandrene and *α*-terpinene are required in order to investigate which of the two volatiles could be more effective in attracting the natural enemy.

3.2 Linking the changes in plant and herbivore biology triggered by beneficial root fungi with the third trophic level functioning

Plant-microbial interactions can influence aboveground indirect plant defenses either by altering plant size/vigor or by modulating plant primary and secondary metabolism (Rasmann et al., 2017). Beneficial root microbes can strongly influence plant growth rate, size, architecture, and vigor (Smith & Read, 2010), which can have negative or positive effects on indirect defenses.

Several studies have reported that more vigorous plants support larger and more vigorous pest populations (Cornelissen et al., 2008), which in turn promote larger parasitoid or predator populations (Kher et al., 2014), that facilitate natural enemy searching efficiency (Andow & Prokrym, 1990; Aslam et al., 2013; Hassell & Southwood, 1978). The transcriptomic analysis conducted in Chapter 2 of this study showed that differentially expressed genes (DEGs) belonging to growth-related categories were upregulated in microbe-inoculated plants in the absence of herbivory. Remarkably, processes related to nutrient uptake were higher regulated in mycorrhizal plants in the absence of herbivory. Therefore, it is hypothesized that the increased nutritional content of the mycorrhizal tomato plants could possibly host a more vigorous population of insect herbivores and would subsequently support and accommodate a more vigorous population of natural enemies. However, measuring the nutritional content of non-inoculated and mycorrhizal tomato plants, and exploring further the potential effects of any resulting nutritional differences on herbivore and natural enemy populations was not into the goals of this study.

Besides plant growth, root mutualistic fungi can also cause changes in primary and secondary plant metabolites in their host plants (Schweiger et al., 2014). These metabolic changes affect not only the performance of the insect herbivores themselves, but also their interactions with predators and parasitoids (Minton et al., 2016). The impacts of beneficial fungi on the interactions between herbivores and their natural enemies qualitatively vary, as the quality of the herbivores (as preys) for their natural enemies can be either enhanced or reduced by the beneficial fungi. As shown in Chapter 1, root mutualistic fungi altered the secondary metabolism of *M. sexta* and the larvae fed upon inoculated plants showed higher oxidative stress. The increased levels of defense metabolites in insect tissues might increase the larval developmental time for non-adapted herbivores and consequently the time available for successful attack by natural enemies, following the “slow-growth-high-mortality” hypothesis proposed by Clancy and Price (1987). As shown in Chapter 1, no significant differences were detected between *M. sexta* larvae fed upon non-inoculated and microbe-inoculated tomato plants regarding the time required for larvae to enter the pre-pupal stage. This result might be explained by the fact that *M. sexta* is better adapted to the secondary metabolites synthesized by plants of the *Solanaceae* family. Nevertheless, at this point, it should be acknowledged that the biological system used in Chapter 3 involved *S. exigua* as the herbivorous insect and not *M. sexta*. *Spodoptera exigua* is a polyphagous, non-adapted insect species fed on a broad range of plants, including also tomato. Therefore, in the same experimental set-up, the developmental time of *S. exigua* larvae reared on beneficial microbe-inoculated plants could hypothetically differ from the developmental time of larvae reared on non-inoculated plants due to an increase in the levels of secondary metabolites. This effect might subsequently positively affect predation/parasitism by natural enemies.

Apart from extending the required developmental time, the increased levels of defense metabolites in insect tissues can reduce the immune capacity of the herbivore hosts, thus

enhancing the chances of successful parasitism (Schmid-Hempel, 2009; Smilanich et al., 2011; Vinson, 1990). However, the enhanced levels of defense metabolites in the tissues of herbivore hosts can also negatively affect their natural enemies. As an example, nicotine incorporated into the diet of *M. sexta* caused statistically significant reductions in the survival of its parasitoid, *Cotesia congregata* (Say) (Barbosa et al., 1986; Thorpe & Barbosa, 1986). Similarly, Harvey et al. (2007) reported that the nicotine contents in the diet of parasitized *M. sexta* L1 larvae significantly affected the cocoon mass of *C. congregata*. Compared to the control diet, parasitoid cocoon mass declined with nicotine in the host diet. The effect was almost linearly correlated with the dietary nicotine concentrations (Harvey et al., 2007). Regarding generalist predators, several studies have described negative consequences on them because of feeding upon preys containing higher levels of secondary metabolites (Malcolm, 1992; Paradise & Stamp, 1990; Stamp et al., 1991; Traugott & Stamp, 1996) as well. On the contrary, a study investigating the consequences of the ingestion of a combination of secondary metabolites, including *α*-tomatine, for *M. sexta* larvae and the generalist stinkbug predator *Podiscus maculiventris* (Say), showed that even if the defense metabolites negatively affected the prey growth, they had no effect on growth, survival, and fecundity of the stinkbug predator (Osier et al., 1996). Interestingly, the authors underlined that a combination of factors may affect the influence of defense metabolites on tritrophic interactions. Such factors include the type (Stamp & Yang, 1996; Traugott & Stamp, 1996) and concentration (Stamp et al., 1991; Traugott & Stamp, 1996) of the secondary metabolites, other defensive metabolites contained in the prey diet (Stamp & Yang, 1996), prey scarcity (Bozer et al., 1996), the age of the predator (Stamp & Yang, 1996) and temperature (Stamp & Yang, 1996; Traugott & Stamp, 1996).

4. The use of beneficial root fungi in Integrated Pest Management for controlling insect pests

Integrated Pest Management (IPM) is a holistic approach to combat pests (including herbivores, pathogens, and weeds) using a combination of preventive and curative actions, and only applying synthetic pesticides when there is an urgent need (Karlsson Green et al., 2020). Since January 2014, all European Union (EU) professional growers are obliged to apply IPM tactics according to the EU Directive Sustainable Use (Directive 2009/128/EC). In this frame, the results of this Doctoral study indicate that the use of root beneficial fungi, such as *R. irregularis* and *T. harzianum*, could serve as a promising tool to protect crop plants even against specialist herbivorous insects. I found that these fungi shaped the plant defense phenotype, including important secondary metabolites protecting plants against herbivores. Moreover, I found important sub-lethal effects associated to induced resistance, including a lower ability of insects to feed from root-inoculated plants and successfully complete their metamorphosis. This could affect the insect populations that attack crops. I further found that the effect of these mutualistic

fungi cascades up to the third trophic level, by affecting the volatile organic compounds emitted by the plant and improving the attraction of a natural enemy.

4.1 The potential of beneficial root fungi in sustainable agriculture: Opportunities and challenges

In analogy to the present study, recent studies using the same model plant and a wide range of beneficial microbes have underlined the ability of beneficial root microbes to protect plants against abiotic and biotic stress factors (Moustaka et al., 2021a, 2021b; Orine et al., 2022; Zitlalpopoca-Hernandez et al., 2022). Within the last decades, several factors have severely affected agricultural production and food security. Among these factors are: 1) the increased frequency and severity of extreme environmental phenomena (i.e. extreme temperatures, drought, high salinity), which are directly associated to the climate change; 2) the intensified demand for food supply due to the ongoing increase of the global population; and 3) the development of insect herbivore and plant pathogen resistance against chemical pesticides, as a result of the uncontrolled application of pesticides in agriculture. Under these circumstances, beneficial root fungi represent a promising alternative to the use of chemical pesticides for sustainable agriculture. Nevertheless, important barriers still prevent us from the wide application of beneficial microbes in agricultural production.

One of these barriers is the high context-dependency that characterizes the plant-microbe interactions (Lee Díaz et al., 2021). This means that microbe-mediated plant resistance against insect herbivores and phytopathogens is triggered when a specific set of conditions is met and is conditional on environmental factors that influence the outcome of plant-microbe interactions (Lee Díaz et al., 2021). Therefore, more interdisciplinary research aiming to uncover the mechanisms that underlie context-dependency in plant-microbe interactions is highly required. Suggested approaches to mitigate the unpredictability in the use of beneficial microbes in agriculture, include: 1) experiments performed under conditions that more realistically mimic the complex set of conditions in agricultural systems (i.e. field experiments); 2) testing targeted sets of environmental conditions (Lee Díaz et al., 2021); and 3) the use of consortia of microbial inoculants (Canfora et al., 2021), and dual or multi-species inoculations to evaluate how microbes interact with each other and with the plant, which is also the case in the agricultural fields (Gadhave et al., 2016; Minchev et al., 2021; Straub et al., 2008; Zitlalpopoca-Hernandez et al., 2022).

On the other hand, although the positive impact of beneficial root microbes on crop protection and production are acknowledged, farmers still perceive these products as challenging to adopt mostly due to their complexity and low observability of positive effects in the field (Ploll et al., 2022). Effective communication strategies, which will bring together different stakeholders, including researchers, farmers, manufacturers, and policy makers, could help to inform farmers

about the positive aspects of beneficial microbe use and accelerate their adoption as part of sustainable agricultural practices.

Summary in English and German

Summary

Plants and insect herbivores have been interacting for several hundred million years. During their co-evolution, they have both evolved strategies to overcome each other's defense mechanisms. The evolutionary arms race between plants and insect herbivores has resulted in the development of a sophisticated defense system that allows plants to recognize herbivore attack and activate plant immune responses to ward off herbivores. Plants confront herbivores both directly and indirectly. Direct plant defenses include physical characteristics that protect plants against herbivores and the production of toxic and/or anti-nutritive compounds that either kill or retard the development of the herbivorous enemies. Indirect defenses include the emission of a blend of volatile compounds that specifically attract the natural enemies of the herbivores, or the provision of food rewards and shelter to enhance the effectiveness of natural enemies. However, in their complex environments, plants also form mutualistic relationships with beneficial soil microbes, such as the arbuscular mycorrhizal fungi (AMF) and plant growth-promoting fungi (PGPF). Beneficial microbes have been long studied for their positive impact on plant growth, nutrition and yield as well as for their plant protective effects against abiotic and biotic stress factors. Nevertheless, more studies involving a combination of transcriptomic and metabolomic approaches are required in order to understand better the mechanisms used by beneficial microbes to induce plant resistance against insect herbivores. In this Doctoral study, I investigated the impact of the beneficial root microbes *Rhizophagus irregularis* and *Trichoderma harzianum* on tomato direct and indirect defense responses against insect herbivores. I used a combination of metabolomic and transcriptomic approaches in order to unravel the molecular and chemical mechanisms that underlie microbe-induced resistance of tomato against herbivores. In Chapter 1 of this dissertation, I aimed to investigate the effect of the two beneficial microbes on the plant and insect metabolome. Moreover, I wanted to answer whether microbe-induced changes in the plant metabolome could cascade up on the metabolome of *Manduca sexta* larvae feeding on the leaves of microbe-inoculated plants. By setting up an insect bioassay, I studied the life cycle of *M. sexta* larvae reared on non-inoculated and microbe-inoculated plants. I observed that root inoculation of tomato with *R. irregularis* and *T. harzianum* impaired *M. sexta* larvae pupation and adult emergence. In order to explain this finding, I used an untargeted metabolomic approach to study: ⅰ) the metabolites accumulated in the leaves of microbe-inoculated plants, and ⅱ) the metabolites accumulated in the gut and fat body tissues of the *M. sexta* larvae fed upon microbe-inoculated plants. The levels of tomato secondary metabolites (*α*-tomatine, *α*-dehydrotomatine) were increased in the leaves of microbe-inoculated plants. In addition, carnitine-derived metabolites, compounds such as piperidine, octanoic acid and benzamide were higher accumulated in the *M. sexta* larvae tissues. Remarkably, the levels of *α*-tomatine were also higher in the gut and fat body tissues of *M. sexta*

larvae reared on microbe-inoculated plants. Therefore, it was concluded that the beneficial microbes altered the metabolome of plants, enhanced the synthesis of defense-related compounds, and this effect cascaded up on the metabolome of the insects fed on microbe-inoculated plants, thereby impairing *M. sexta* physiology and life cycle. In Chapter 2 of this dissertation, I pursued an integrated -omics approach in order to address the impact of the two beneficial microbes on the transcriptomic and metabolomic response of tomato to *M. sexta* herbivory. Therefore, I used a high-throughput RNA sequencing and metabolomic analysis, followed by the combination of the two approaches. The transcriptomic analysis separated non-infested from herbivore-infested samples. Herbivory was correlated with the downregulation of genes involved in photosynthesis and the upregulation of genes involved in stress responses. In response to herbivory, genes involved in secondary metabolism were upregulated in *T. harzianum*-inoculated plants. In parallel, genes involved in stress-related responses were upregulated in *R. irregularis*-inoculated plants. The metabolomic analysis clearly separated non-infested from herbivore-infested samples as well. Interestingly, the integrative analysis, performed over herbivory samples, separated the samples based on the beneficial microbe used. The correlation network analysis, performed over the same dataset, resulted in the formation of two correlation sub-networks, Rhi-focused and Tri-focused, enriched in elements modulated by the presence of *R. irregularis* and *T. harzianum*, respectively. In Chapter 3 of this dissertation, I aimed to investigate the impact of the two beneficial microbes on the induction of indirect defenses in response to herbivory by *Spodoptera exigua* larvae. With this aim, I initially tested the attraction of the zoophytophagous predator *Macrolophus pygmaeus* towards non-inoculated and microbe-inoculated herbivore-infested plants. Furthermore, I analyzed the herbivore-induced volatiles emitted by non-inoculated and microbe-inoculated plants as well as the expression levels of genes involved in the synthesis of herbivore-induced volatiles. It was observed that among herbivore-infested plants, *M. pygmaeus* preferred the microbe-inoculated compared to the non-inoculated ones. In addition, inoculation with beneficial microbes had a marginal effect on the emission of specific terpenoids that might affect the choice of *M. pygmaeus*. However, the gene expression analysis showed that the interaction effect between herbivory and beneficial microbes significantly affected the expression levels of only one marker gene. In conclusion, the results presented in this PhD study indicate that the beneficial root microbes *R. irregularis* and *T. harzianum* mediate plant-insect interactions and can trigger important effects over the three trophic levels: the plant, its herbivores, and their natural enemies. Thus, these findings strengthen the potential of beneficial root fungi to be used in agriculture as a promising alternative tool to reduce the use of chemical insecticides, ensure crop productivity and food security, and protect human health as well as the environment.

Zusammenfassung

Pflanzen und Insektenfresser stehen seit mehreren hundert Millionen Jahren in Wechselwirkung. Während ihrer gemeinsamen Evolution haben beide Strategien entwickelt, um die Abwehrmechanismen des jeweils anderen zu überwinden. Das evolutionäre Wettrüsten zwischen Pflanzen und Insektenfressern hat zur Entwicklung eines ausgeklügelten Abwehrsystems geführt, das es den Pflanzen ermöglicht, Angriffe von Pflanzenfressern zu erkennen und pflanzliche Immunreaktionen zur Abwehr von Pflanzenfressern zu aktivieren. Pflanzen sind sowohl direkt als auch indirekt mit Pflanzenfressern konfrontiert. Zu den direkten pflanzlichen Abwehrmechanismen gehören physische Merkmale, die die Pflanzen vor Pflanzenfressern schützen, sowie die Produktion von toxischen und/oder nährstofffeindlichen Verbindungen, die Entwicklung der pflanzenfressenden Feinde entweder töten oder verzögern. Zu den indirekten Abwehrmechanismen gehören die Emission einer Mischung flüchtiger Verbindungen, die speziell die natürlichen Feinde der Pflanzenfresser anlockt, oder die Bereitstellung von Nahrung und Unterschlupf, um die Wirksamkeit der natürlichen Feinde zu erhöhen. In ihrer komplexen Umwelt gehen Pflanzen jedoch auch mutualistische Beziehungen mit nützlichen Bodenmikroben wie den arbuskulären Mykorrhizapilzen (AMF) und pflanzenwachstumsfördernden Pilzen (PGPF) ein. Nützliche Mikroben werden seit langem wegen ihrer positiven Auswirkungen auf Pflanzenwachstum, Ernährung und Ertrag sowie wegen ihrer pflanzenschützenden Wirkung gegen abiotische und biotische Stressfaktoren untersucht. Dennoch sind weitere Studien erforderlich, die eine Kombination aus transkriptomischen und metabolomischen Ansätzen beinhalten, um die Mechanismen besser zu verstehen, die von nützlichen Mikroben genutzt werden, um Pflanzenresistenz gegen Insektenherbivoren zu erzeugen. In dieser Doktorandenstudie untersuchte ich die Auswirkungen der Nutzmikroben *Rhizophagus irregularis* und *Trichoderma harzianum* auf die direkten und indirekten Abwehrreaktionen von Tomaten gegen Insektenherbivoren. Ich verwendete eine Kombination aus metabolomischen und transkriptomischen Ansätzen, um die molekularen und chemischen Mechanismen zu entschlüsseln, die der mikrobeninduzierten Resistenz der Tomate gegen Pflanzenfresser zugrunde liegen.

In Kapitel 1 dieser Dissertation wollte ich die Auswirkungen der beiden nützlichen Mikroben auf das Metabolom von Pflanzen und Insekten untersuchen. Darüber hinaus wollte ich herausfinden, ob die durch die Mikroben hervorgerufenen Veränderungen im Metabolom der Pflanze sich auch auf das Metabolom von *Manduca sexta*-Larven auswirken, die sich von den Blättern der mit Mikroben beimpften Pflanzen ernähren. Mit Hilfe eines Insekten-Bioassays untersuchte ich den Lebenszyklus von *M. sexta*-Larven, die auf nicht inokulierten und mikrobeninokulierten Pflanzen aufgezogen wurden. Ich stellte fest, dass die Inokulation von Tomatenwurzeln mit *R. irregularis* und *T. harzianum* die Verpuppung und den Schlupf von *M. sexta*-Larven beeinträchtigte. Um diesen Befund zu erklären, habe ich einen ungezielten metabolomischen Ansatz zur Untersuchung verwendet: I) die Metaboliten, die sich in den Blättern der mit Mikroben

geimpften Pflanzen ansammelten, und II) die Metaboliten, die sich im Darm und im Fettgewebe der *M. sexta*-Larven ansammelten, die sich von mit Mikroben geimpften Pflanzen ernährten. Der Gehalt an Sekundärmetaboliten der Tomate (*α*-Tomatin, *α*-Dehydrotomatin) war in den Blättern der mit Mikroben beimpften Pflanzen erhöht. Darüber hinaus wurden von Carnitin abgeleitete Metaboliten, Verbindungen wie Piperidin, Octansäure und Benzamid in den Geweben der *M. sexta*-Larven stärker angereichert. Bemerkenswerterweise war auch der Gehalt an *α*-Tomatin im Darm- und Fettkörpergewebe von *M. sexta*-Larven, die auf mit Mikroben beimpften Pflanzen aufgezogen wurden, höher. Daraus wurde gefolgert, dass die nützlichen Mikroben das Metabolom der Pflanzen veränderten, die Synthese von abwehrrelevanten Verbindungen erhöhten und dieser Effekt sich kaskadenartig auf das Metabolom der Insekten auswirkte, die mit mikrobeninokulierten Pflanzen gefüttert wurden, wodurch die Physiologie und der Lebenszyklus von *M. sexta* beeinträchtigt wurden.

In Kapitel 2 dieser Dissertation verfolgte ich einen integrierten -omics-Ansatz, um die Auswirkungen der beiden nützlichen Mikroben auf die transkriptomische und metabolomische Reaktion der Tomate auf die Herbivorie von *M. sexta* zu untersuchen. Dazu verwendete ich eine Hochdurchsatz-RNA-Sequenzierung und eine Metabolomanalyse, gefolgt von einer Kombination der beiden Ansätze. Die transkriptomische Analyse trennte nicht befallene von durch Herbivoren befallenen Proben. Herbivorie korrelierte mit der Herunterregulierung von Genen, die an der Photosynthese beteiligt sind, und der Hochregulierung von Genen, die an Stressreaktionen beteiligt sind. Als Reaktion auf Herbivorie wurden Gene, die am Sekundärstoffwechsel beteiligt sind, in mit *T. harzianum* beimpften Pflanzen hochreguliert. Parallel dazu wurden Gene, die an Stressreaktionen beteiligt sind, in mit *R. irregularis* beimpften Pflanzen hochreguliert. Die metabolomische Analyse trennte auch eindeutig zwischen nicht befallenen und von Herbivoren befallenen Proben. Interessanterweise trennte die integrative Analyse, die anhand der Herbivorenproben durchgeführt wurde, die Proben auf der Grundlage der verwendeten nützlichen Mikrobe. Die Analyse des Korrelationsnetzes, die mit demselben Datensatz durchgeführt wurde, ergab zwei Korrelations-Subnetze, die mit Elementen angereichert waren, die durch die Anwesenheit von *R. irregularis* und *T. harzianum* moduliert wurden.

In Kapitel 3 dieser Dissertation wollte ich die Auswirkungen der beiden nützlichen Mikroben auf die Induktion indirekter Abwehrmechanismen als Reaktion auf Herbivorie durch *Spodoptera exigua*-Larven untersuchen. Zu diesem Zweck testete ich zunächst die Anziehungskraft des zoophytophagen Räubers *Macrolophus pygmaeus* auf nicht beimpfte und mit Mikroben beimpfte Pflanzen, die von Herbivoren befallen waren. Darüber hinaus analysierte ich die von Pflanzenfressern induzierten flüchtigen Stoffe, die von nicht beimpften und mit Mikroben beimpften Pflanzen abgegeben werden, sowie die Expressionsniveaus von Genen, die an der Synthese von Pflanzenfresser-induzierten flüchtigen Stoffen beteiligt sind. Es wurde festgestellt, dass *M. pygmaeus* unter den von Herbivoren befallenen Pflanzen, die mit Mikroben geimpften gegenüber den nicht geimpften Pflanzen bevorzugte. Darüber hinaus hatte die Inokulation mit nützlichen Mikroben einen marginalen Einfluss auf die Emission spezifischer Terpenoide, die

Wahl von *M. pygmaeus* beeinflussen könnten. Die Analyse der Genexpression zeigte jedoch, dass der Interaktionseffekt zwischen Herbivorie und nützlichen Mikroben das Expressionsniveau von nur einem Markergen signifikant beeinflusste. Die in dieser Doktorarbeit vorgestellten Ergebnisse deuten darauf hin, dass die nützlichen Wurzelmikroben *R. irregularis* und *T. harzianum* Wechselwirkungen zwischen Pflanzen und Insekten vermitteln und wichtige Auswirkungen auf die drei trophischen Ebenen haben können: die Pflanze, ihre Herbivoren und ihre natürlichen Feinde. Diese Ergebnisse untermauern das Potenzial der nützlichen Wurzelpilze, in der Landwirtschaft als vielversprechende Alternative eingesetzt zu werden, um den Einsatz chemischer Insektizide zu verringern, die Produktivität der Pflanzen und die Ernährungssicherheit zu gewährleisten und die menschliche Gesundheit sowie die Umwelt zu schützen.

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Declaration of honor

I hereby confirm that:

I. I am familiar with the valid doctoral examination regulations.

II. I produced the present doctoral thesis myself (statement of authorship). I neither used any text passages from third parties, nor my own previous final theses without citing those: in addition, I also confirm that I have cited the tools, personal information, and the sources that have been used for the production of the present doctoral thesis.

III. While working on the experiment described in Chapter 1 of this doctoral thesis, I was supervised and mentored by Dr. Ainhoa Martínez-Medina to set up my experiments. Dr. Alexander Weinhold supported me in the analysis insect metabolites and Dr. Fredd Vergara supported me in the analysis of plant metabolites. Prof. Søren Bak hosted me in his laboratories and group in the University of Copenhagen in order to inject insect metabolite samples. There, I also collaborated with Dr. Bekzod Khakimov and Dr. David Ian Pattison while injecting my samples and acquiring the raw spectral data. The manuscript was written by me with the assistance of Prof. van Dam and Dr. Ainhoa Martínez-Medina. While working on the experiment described in Chapter 2 of this doctoral thesis, I was supervised and mentored by Dr. Ainhoa Martínez-Medina to set up my experiments. Furthermore, I was assisted by Dr. Fredd Vergara in the analysis of plant metabolites and by Dr. Francisco Colina in the bioinformatics analyses. The thesis chapter was written by me and reviewed by Dr. Ainhoa Martínez-Medina. While working on the experiment described in Chapter 3 of this doctoral thesis, I set up my experiments with the help of Ms. Dongik Chang that I was supervising on her Master study. I was assisted by Dr. Alexander Weinhold in the analysis of volatile compounds. The first draft of the manuscript was written by me and reviewed by Dr. Alexander Weinhold.

IV. I did not receive any assistance from specialized consultants and no third party received either direct or indirect financial benefits from me for the work connected to the doctoral thesis submitted.

V. I have not already submitted the doctoral thesis project as my final thesis for a state examination or other scientific examination.

VI. I have not submitted the same, or substantially similar, or another scientific paper to any other institution of higher education or to any other faculty.

Appendix

FORM 2

Manuscript No. 1

Short reference: Papantoniou et al (2021), Metabolites

Contribution of the doctoral candidate

Contribution of the doctoral candidate to figures reflecting experimental data (only for original articles):

Figure #1	☐	100% (the data presented in this figure come entirely from experimental work carried out by the candidate)
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Figure # 2	☐	100% (the data presented in this figure come entirely from experimental work carried out by the candidate)
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	☑	Approximate contribution of the doctoral candidate to the figure: 90 % Brief description of the contribution: *(The doctoral candidate ground the leaf material, extracted the metabolites, analyzed the data and composed the figure)*
Figures # 3-6	☑	100% (the data presented in this figure come entirely from experimental work carried out by the candidate)
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FORM 2

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FORM 2

Manuscript No. 3

Short reference Papantoniou et al (2022), Frontiers in Physiology, section Invertebrate Physiology

Contribution of the doctoral candidate

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