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BENEFICIAL FUNGI MODULATE HERBIVORE-INDUCED PLANT DEFENSES OF ARABLE CROPS IN MULTITROPHIC INTERACTIONS

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Summary

Plants are the primary producers of most terrestrial ecosystems and are constantly threatened by a wide range of attackers, including herbivorous insects. It has been known that insect herbivores are important antagonists in agricultural systems, causing considerable yield losses. In agriculture, insect pests have often been managed by the indiscriminate application of chemical insecticides. However, most of these insecticides can cause serious hazards to biodiversity, to the environment and, in addition, they could become ineffective due to development of pest resistance. Thus, there is an increased interest in finding effective and environmentally friendly alternatives to chemical insecticides, which include the use of natural enemies of herbivore pests, biopesticides and, more recently, beneficial microorganisms.

Beneficial microbes provide several benefits to plants by promoting growth, enhancing nutrient uptake or suppressing plant pathogens and herbivores. They can also prime plant defenses for stronger resistance and increase the attraction of the natural enemies toward plants under herbivore attack. Although there have been several attempts to elucidate the complex interaction between plants, beneficial microbes and herbivores from different feeding guilds, there is no information available on the impacts of beneficial microbes mediating plant defense responses against herbivore stink bugs.

In this thesis, I studied whether and how beneficial microbes help the plants by enhancing plant defenses in response to stink bug attacks. The model study organisms evaluated here consists of two beneficial microbes, the plant-growth promoting fungi *Trichoderma harzianum* strain T22 and the entomopathogenic fungi *Beauveria bassiana* ARSEF 3097; two plants species, tomato *Solanum lycopersicum* and *Capsicum annuum*; two major herbivore stink bugs species, *Nezara viridula* and *Halyomorpha halys* and their two associated egg parasitoids, *Trissolcus basalis* and *Trissolcus japonicus*. The aim of the thesis was to gain a better understanding of beneficial microbes' ability to influence direct and indirect plant defenses in response to *N. viridula* and *H. halys* damages.

Results in Chapter 1 have shown the effects of *T. harzianum* T22 colonization on tomato direct plant defenses against feeding by the southern green stink bug *N. viridula*. It has been observed that tomato root colonization by *T. harzianum* T22 resulted in the decreased relative growth rate of *N. viridula* nymphs compared to non-inoculated plants. Furthermore, the crucial role of the Jasmonic Acid (JA)-defense signaling pathway in regulating *Trichoderma*-mediated defenses against *N. viridula* is also confirmed by plant defense-related gene expression analysis. At the molecular level, it has been demonstrated that tomato plants respond to *N. viridula* feeding by activating the JA-defense signaling

pathway detected through an increase in expression levels of defense marker genes *ToLOX D* and *ToPIN2* after 8h of stink bug feeding. This early activation of the JA-defense signaling pathway in inoculated plants revealed that *T. harzianum* T22 primed tomato plants which responded faster and more effectively to stink bug feeding. To conclude, these results indicate that the strength of plant responses to stink bug feeding is positively affected by root inoculation with *T. harzianum* T22.

In Chapter 2, I focused on how plant defenses induced by insect egg deposition can be modulated by beneficial microbes. In this context, I used the following multitrophic systems (i) tomato plant – *T. harzianum* T22 – *N. viridula* – *T. basalis*; (ii) sweet pepper plant – *T. harzianum* T22 – *N. viridula* – *T. basalis*; (iii) sweet pepper plant – *B. bassiana*– *N. viridula* – *T. basalis*. Results of this chapter showed that inoculation by *T. harzianum* T22 affected the blend of volatile organic compounds emitted from tomato plants induced by stink bug feeding and oviposition, resulting in an increased attraction of the egg parasitoid *T. basalis*. However, in contrast to the outcome of *T. harzianum* T22, endophytic colonization of *B. bassiana* in sweet pepper plants induced by stink bug feeding and oviposition decreased attraction of *T. basalis* as female wasps preferred non-inoculated plants over inoculated ones. Within this chapter, it has been concluded that inoculating tomato and sweet pepper plants with *T. harzianum* T22 enhances indirect plant defenses in response to *N. viridula* feeding and oviposition activity. Yet *B. bassiana* negatively affects the attraction of egg parasitoids to the infested sweet pepper plants.

Chapter 3 addressed how colonization by *T. harzianum* T22 affects direct and indirect plant defenses against the brown marmorated stink bug *H. halys* by combining chemical, gene transcriptional and behavioral approaches. In a first set of bioassays, I found that *H. halys* nymphs which developed on *T. harzianum* T22 inoculated tomato plants exhibited a lower weight compared with nymphs that developed on non-inoculated plants. According to transcript levels of defense marker genes, root colonization by *T. harzianum* T22 resulted in an up-regulation of the salicylic acid (SA)-related gene *ToPR1* after 72h of stink bug feeding and the JA-dependent gene *ToPIN2* after 8h. Furthermore, when the both plants were induced by *N. viridula* oviposition, the egg parasitoid *T. japonicus* was more attracted to *Trichoderma*-inoculated plants compared to non-inoculated plants. Overall, this chapter shows that *T. harzianum* T22 has a high potential in the biocontrol of *H. halys* based on both enhanced direct and indirect plant defenses.

Taken together, the research presented in this thesis illustrates how beneficial microbes mediate direct and indirect plant defenses in a context-depended manner which depends on the specificity of the

interactions among plants, microbes and insects. The findings of this thesis are particularly relevant for the use of beneficial microbes in agriculture, as microbial biocontrol might be an effective tool for controlling insect herbivores in an environmentally friendly manner.

1. Introduction

Plants live in complex environments where they interact with a multitude of antagonistic organisms, such as insect herbivores and plant pathogens. Because plants are sessile organisms, they may initially be perceived as vulnerable targets, especially for the wide variety of herbivores. Herbivores cause an estimated 18% to 25% of agricultural productivity losses worldwide, with tremendous losses occurring in regions with food shortage (Savary et al., 2019).

Insect herbivore damages in agricultural systems result in significant losses in yield due to decreased crop productivity and storage capacity (Oerke, 2006; Culliney, 2014; Kumar & Kalita, 2017). During the last decades, the uncontrolled and massive use of chemical insecticides to control insect pests has led to severe environmental damage and major health concerns (Tilman et al., 2002; Pimentel, 2005). Therefore, there is an increased global interest in discovering alternative strategies to chemical pesticides that can be employed in modern agriculture. In this context, different environmentally friendly alternatives are being developed, such as the use of natural enemies of herbivore pests, biopesticides and, more recently, beneficial microorganisms (DeBach, 1964; Flint & Dreistadt, 1998; Van Lenteren et al., 2018).

Plants interact with a broad community of mutualistic microbes that provide them with important benefits (Guerrieri & Digilio, 2008; Pieterse et al., 2014; Bender et al., 2016). The beneficial microbes include several microorganisms such as arbuscular mycorrhizal fungi (AMF), endophytic fungi (EF), plant growth-promoting rhizobacteria (PGPR) and plant growth-promoting fungi (PGPF). The key benefits are associated with the establishment of a protective microbial communities, improvement of plant health, growth promotion, increased nutrient availability and uptake (Bezemer & van Dam, 2005; Vinale et al., 2008; Lugtenberg & Kamilova, 2009; Berendsen et al., 2012). It is well demonstrated that improved nutritional status appears to be associated with a reduced incidence of plant pathogen adverse effects on plant (Dordas, 2008). Furthermore, beneficial microbes are also capable of suppressing pathogens locally or systemically through induced systemic resistance (ISR) and systemic acquired resistance (SAR) (Harman et al., 2004; Partida-Martinez & Heil, 2011). The ISR and SAR are regulated by jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) defense signaling pathways (Kessler et al., 2004; Howe & Jander, 2008; Van Oosten et al., 2008) which are used by plants against a wide range of plant pathogens (van Wees et al., 2008; Zamioudis & Pieterse, 2012; Pieterse et al., 2014). For instance, the PGPR

Pseudomonas fluorescens triggers ISR against infection by the bacterial leaf pathogen *P. syringae* pv *tomato* by activating JA and ET defense signaling pathways in *Arabidopsis* plants (Pieterse et al., 1998). ISR is also linked with insect herbivores in terms of triggered defense responses which inhibits the insect's digestive enzymes, alters the nutritional value of the food plant and recruits natural enemies (Dicke & Van Poecke, 2002; Gatehouse, 2002; Vallad & Goodman, 2004). In fact, beneficial microbe interactions with plants against insect herbivores depend on isolate-specific traits (Harman, 2004) and this interaction might vary in the eventual outcome as positive, negative or neutral effects to plants (Pozo et al., 2013; McKinnon et al., 2017; Poveda, 2021). Nevertheless, most of recent findings have shown that beneficial microbes can mediate plant defenses, leading to negative effects on aboveground and belowground herbivores (Gehring & Bennett, 2009; Shoresh et al., 2010; Pineda et al., 2010; Pieterse et al., 2014; Sugio et al., 2015; Rasmann et al., 2017; Pappas et al., 2018) (Fig 1.).

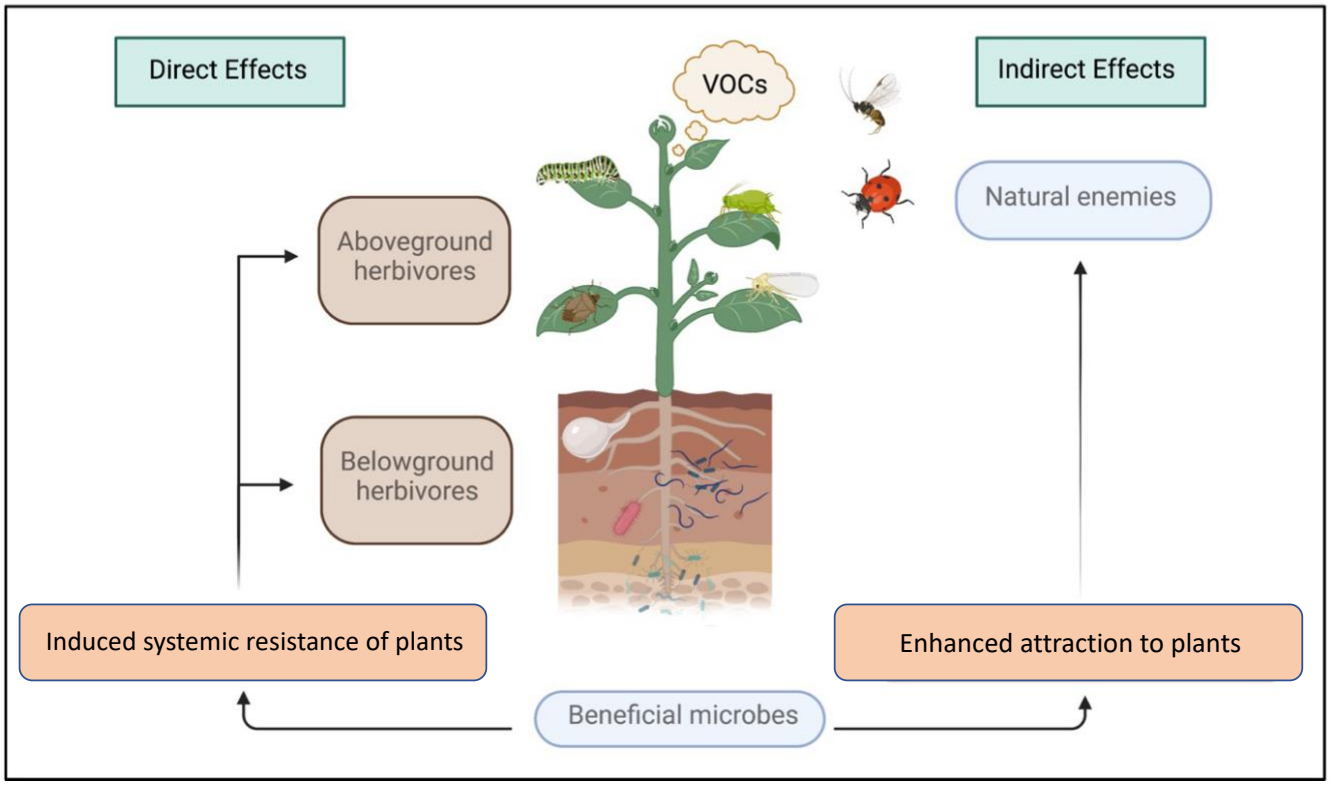

Fig. 1. General outcomes of interactions among beneficial microbes, plants and insect species

Beneficial microbes affect plant defenses against herbivores in several ways. First, they trigger plant defenses, prime plants and produce insecticidal metabolites that directly affect herbivores (Pieterse et al., 2014). For instance, root inoculation of *Arabidopsis* plants with *P. fluorescens* WCS417r was found to

reduce the larval weight of cabbage moth *Mamestra brassicae* L. (Lepidoptera: Noctuidae) (Pangesti et al., 2015). The authors suggested that this effect occurred through the increased expression of JA-dependent gene *LOX2* and the JA- and ET-dependent genes *PDF1.2* and *HEL*. Yet another beneficial microbe, the PGPF *Trichoderma atroviride*, was found to reduce foliar damage caused by the fall armyworm *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae) via effects on the JA-signaling pathway (Contreras-Cornejo et al., 2018a). In a study carried out by Pappas et al. 2018, colonization by endophytic fungus *Fusarium solani* strain K suppressed the two spotted-spider mite *Tetranychus urticae* Koch (Acari: Tehranychidae) performance on tomato plants. Another study demonstrated that inoculating the leaves of *Cirsium arvense* and *Arabidopsis thaliana* with *T. viride* and *T. gamsii* reduced the feeding damage by thistle tortoise beetles, *Cassida rubiginosa* Müller (Coleoptera: Chrysomelidae) (Gange et al., 2012).

Beneficial microbes can induce a state of alert (called "defense priming") in plants that results in a more effective defense response against future herbivores (Lorito et al., 2010; Hermosa et al., 2012; Conrath et al., 2015). For instance, colonization of tomato plants by AMF, *Glomus mosseae*, can prime systemic defense responses against the corn earworm *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) by increasing the expression of the defense-associated genes allene oxide cyclase (*AOC*), lipoxygenase (*LOXD)* and protease inhibitors (*PI-I, PI-II*) (Song et al., 2013). A more recent study suggests that the PGPF *T. harzianum* T78 limited damage inflicted by the root-knot nematode *Meloidogyne incognita* (Kofoid & White) Chitwood (Tylenchida: Heteroderidae) through priming the SA- and the JA- pathways in tomato roots (Martínez-Medina et al., 2017).

Besides enhancing direct plant defenses, beneficial microbes can enhance indirect plant defenses by recruiting natural enemies of the attacking herbivores (Rasmann et al., 2017). During feeding and/or oviposition by herbivorous insects, plants exhibit changes in their physiological characteristics and emit specific volatile organic compounds (VOCs), which are called herbivore-induced plant volatiles (HIPVs) or oviposition-induced plant volatiles (OIPVs) (Hilker & Fatouros, 2015). Inoculation of *T. longibrachiatum* MK1 in tomato plants attacked by the potato aphid *Macrosiphum euphorbiae* (Thomas) (Hemiptera: Aphididae) modified the release of specific HIPVs which resulted in a higher attractiveness toward the aphid parasitoid *Aphidius ervi* Haliday (Hymenoptera: Braconidae) and the aphid predator *Macrolophus pygmaeus* Rambur (Hemiptera: Miridae) (Battaglia et al., 2013). In another study system it was shown that the interaction between PGPR *Bacillus amyloliquefaciens*, plants and lepidopteran herbivores altered the behavior of the predatory earwig *Doru luteipes* Scudder (Dermaptera:

Forficulidae) (Bell et al., 2020). In this study, *D. luteipes* was more attracted to PGPR inoculated plants over non-inoculated plants when leaf damage by caterpillars of *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) and *S. frugiperda* occurred.

To date, several studies have investigated the effect of beneficial microbes in multitrophic interactions. Studying interactions in the environment is crucial for understanding ecosystem services, interplays among organisms and potential biocontrol agents interacting at different trophic levels. Further research is needed to determine whether beneficial microbes whose efficiency depends on plant-herbivore-natural enemy species-specific traits, can be incorporated into multiple trophic systems. The novel findings would contribute to knowledge necessary to extend the usage of beneficial microbes in biological control programs.

2. Main objective and research questions

The main objective of my PhD research was to further be understanding on mechanisms involved in the interactions among beneficial microbes, plants, herbivores and their associated natural enemies. While several multitrophic studies considering different feeding guilds of herbivores have been reported up to now, it is still unclear how beneficial microbes modulate plant defense responses against piercing-sucking herbivores such as stink bugs. To fill this gap, I have used an integrated approach combining behavioral assays, gene transcriptional and chemical approaches in order to reveal the role of beneficial microbes in direct and indirect plant defenses against stink bugs. Following research questions have addressed:

1) How do beneficial fungi affect direct plant defenses against stink bugs?
    a) What are the effects of beneficial fungi on the performance of stink bugs nymphs feeding on inoculated plants?
    b) Does inoculation of beneficial fungi regulate defense signaling pathways in response to stink bugs?
2) How do beneficial fungi alter indirect plant defenses against stink bugs?
    a) Is the attraction of egg parasitoids increased by the colonization of beneficial fungi?
    b) Can beneficial fungi modulate profile of VOCs?
    c) What are the underlying chemical mechanisms of the indirect plant defenses enhanced by beneficial fungi?

3. Study systems

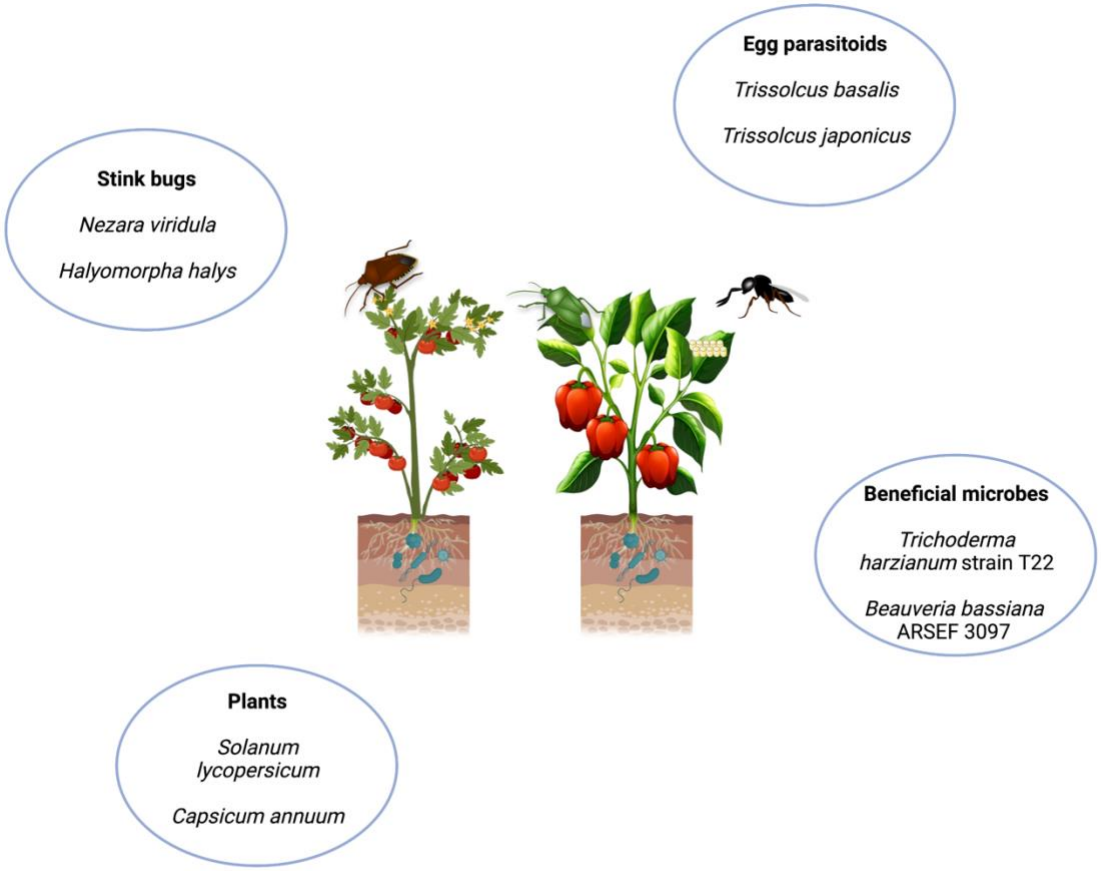

Fig. 2. Representation of the model systems

3.1. Beneficial microbes

In this thesis, I have selected two beneficial microbe species involved in different groups, the entomopathogenic fungus *Beauveria bassiana* ARSEF 3097 (ATCC 74040) and the plant-growth promoting fungus *T. harzianum* strain T22. Besides their usage as bioinsecticides on bodies of herbivores (e.g., directly spraying), I here focused on the plant colonization of these two beneficial fungi as direct spraying may limit fungal effectiveness due to their susceptibility to ultraviolet light (UV) and low moisture content (Vega, 2018).

3.1.1. Entomopathogenic fungus, *Beauveria bassiana*

Beauveria bassiana strains are common terrestrial entomopathogens found to occur endophytically in several plant species and, in addition, they have been artificially inoculated in various important crop

plants (Vega, 2008; Akutse et al., 2013; Jaber & Enkerli, 2017). Plant endophytic colonization by entomopathogenic fungi was first reported in maize (*Zea mays* L.) where it was discovered that 33% of maize plants were naturally colonized by *B. bassiana*, while artificial inoculations achieved higher levels of colonization up to 98.3% (Bing & Lewis, 1991, 1992). Endophytic colonization by *B. bassiana* can promote plant growth (Jaber, 2018) and enhance plant defenses against plant pathogens (Ownley et al., 2004, 2008). Moreover, in several studies it has been demonstrated that *B. bassiana* has either a negative or no effect on insect herbivores when act as a plant endophyte (McKinnon et al., 2017; Vega, 2018; Raad et al., 2019; Fingu-Mabola et al., 2020; Wilberts et al., 2022). The main negative effects were reported to be on the reproduction of herbivores (McKinnon et al., 2017). For instance, the population growth of the pea aphid *Acyrthosiphon pisum* (Harris) (Hemiptera: Aphididae) was reduced when they fed on the faba bean plants colonized by *B. bassiana* (Akello & Sikora, 2012). Same outcomes were also observed for another aphid species, *Aphis gossypii* Glover (Hemiptera: Aphididae), feeding on *B. bassiana* inoculated cotton plants (Gurulingappa et al., 2010).

However, the impact of *B. bassiana* on herbivore performance is well complex. In fact, a recent study involving two generations of experiments found that colonization of *B. bassiana* strain GHA in *Vicia faba* L. plants did not affect the fertility of black bean aphids *Aphis fabae* Scopoli (Hemiptera: Aphididae) in the first generation, but in the second generation more nymphs were found on inoculated plants compared to non-inoculated plants (Jensen et al., 2019).

The effect of B. bassiana *on direct plant defenses*

It has been demonstrated that tobacco seedlings colonized by *B. bassiana* strains were more resistant to the green peach aphid *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) and activated both the JA and SA defense pathways (Qin et al., 2021). A study by Jensen et al. 2020 showed that seed inoculation of *B. bassiana* triggered the expression of *PR1*, a marker gene of SA-pathway after 72 hours of *A. fabae* infestation on broad bean plants. Furthermore, the transcript level of *ERF-1*, a gene involved in the ET-pathway was also increased after 24 hours (Jensen et al., 2020). *Beauveria bassiana* was found to alter HIPV emission when the tomato plants were infested by caterpillars of the beet armyworm *S. exigua*. Volatile analysis showed that higher levels of two monoterpenes (δ-2- carene, sabinene) and three sesquiterpenes (δ-elemene, (E)-β-caryophyllene, α-humulene) were emitted by *B. bassiana* colonized plants compared to control ones (Shrivastava et al., 2015).

The effect of B. bassiana *on indirect plant defenses*

There is limited information available regarding the effects of endophytic colonization on indirect plant defenses. In fact, so far, most studies have focused on the safety usage of *B. bassiana* when directly interacting with natural enemies (e.g., side effects etc.) (Labbé et al., 2009; Rashki et al., 2009; Akutse et al., 2014; Bajya et al., 2015; Jaber & Araj, 2018; Canassa et al., 2019). Nevertheless, Jensen et al., 2020 showed that *B. bassiana* colonization on bean plants can affect the choice behavior and fitness of the aphid parasitoid, *Aphidius colemani* Viereck (Hymenoptera: Aphidiidae). The authors reported that the number of parasitized aphids were not different between inoculated and control plants; however, the emergence of adult parasitoids was significantly lower when they emerged from aphids that infested *B. bassiana* colonized plant. Remarkably, in a choice bioassay, larvae of the generalist predator *Chrysoperla carnea* (Steph.) (Neuroptera: Chrysopidae) preferred to feed on the cotton aphid *A. gossypii* on *B. bassiana*-colonized plants compared to control plants (González-Mas et al., 2019). González-Mas et al., 2021 showed that endophytic colonization by *B. bassiana* strain EABb 01/33-Su can change the HIPV profile of melon and cotton plants induced by *A. gossypii* or by *Spodoptera* spp. caterpillars. According to the authors, some of the distinct compounds found in the blend of VOCs have been reported previously as potential attractants to natural enemies, yet their possible role in the recruitment of specific natural enemies remains to be confirmed.

3.1.2. Plant- growth promoting fungus, *Trichoderma harzianum*

Trichoderma harzianum is one of commercially available biological control agent holding potential for sustainable crop production (Vitti et al., 2015). *Trichoderma harzianum* strains are widespread soilborne fungi found in soils from tropical to temperate environments and are able to survive well in a wide range of soil conditions (Gams & Meyer, 1998; Delgado-Jarana et al., 2006; Mukherjee et al., 2013; Leelavathi et al., 2014). They have traditionally been considered common soil saprophytes; however, research has shown that they can form opportunistic avirulent symbiotic relationships with plant roots (Ahmad & Baker, 1987; Druzhinina et al., 2011). Currently, the different strains are well known for their beneficial effects on plant growth, resistance to abiotic stresses and ability to perform mycoparasitism on plant pathogens (Elad et al., 1980; Kleifeld & Chet, 1992; Yedidia et al., 2001; Harman et al., 2004; Woo et al., 2014). Although they have been mostly considered as effective control agents against plant pathogens, recent studies have revealed their role in mediating both direct and indirect plant defenses against herbivores (Battaglia et al., 2013; Coppola et al., 2019; Menjivar et al., 2012; Muvea et al., 2014).

The effect of T. harzianum *on direct plant defenses*

To date, a few studies have investigated the role of *T. harzianum* on direct plant defenses against insect herbivores. In one of these studies, the activation of SA-mediated defenses in tomato plants colonized by *T. harzianum* has been found to increase the mortality of whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) by up to 35% (Jafarbeigi et al., 2020). Similar results were also obtained for the cell-content feeder *Thrips tabaci* Lindeman (Thysanoptera: Thripidae) as onion plants colonized by *T. harzianum* ICIPE 709 suffered reduced feeding punctures and fewer number of eggs laid (Muvea et al., 2014).

Evidence suggests that *T. harzianum* can enhance plant defenses by inducing ISR and SAR (Shoresh et al., 2010). Furthermore, plant colonization by *T. harzianum* activates the JA and SA defense signaling pathways (Korolev et al., 2008; Shoresh et al., 2005). Coppola et al., 2019 found that colonization of tomato plants by *T. harzianum* strain T22 had a negative effect on the lifespan of aphids *M. euphorbiae* which is likely explained by an induction of a JA-related priming state against the aphid.

ISR is a mechanism by which *Trichoderma* can also prime plant defenses in response to insect herbivores (Harman et al., 2004; Nawrocka & Małolepsza, 2013). As a consequence of the regulation of ISR by *Trichoderma*, the plant can establish a state of alert that makes the plant more capable to defend itself against herbivory (Martínez-Medina et al., 2017). The primed plant can activate a more rapid and intense response to biotic stressors (Conrath, 2009; Lorito et al., 2010; Van der Ent et al., 2009). For example, a priming effect due to enhanced SA-defenses was demonstrated to be induced by *T. harzianum* T78 in tomato plants which limited root invasion of nematode *M. incognita* (Martínez-Medina et al., 2017).

The effect of T. harzianum *on indirect plant defenses*

Trichoderma species can influence indirect plant defenses through modulation of VOC emissions (Coppola et al., 2017; Contreras-Cornejo et al., 2018b; Parrilli et al., 2019). For instance, *T. harzianum* strain T22 modulates VOCs emitted by tomato plants leading to enhanced attraction of the aphid parasitoid *A. ervi* (Coppola et al., 2017). Furthermore, maize plants infested by *S. frugiperda* and colonized by *T. atroviride* have been found to be more attractive for natural enemy of *S. frugiperda, Campoletis sonorensis* (Cameron) (Hymenoptera: Ichneumonidae) (Contreras-Cornejoa et al., 2018b).

3.2. Plants

3.2.1. Tomato, *Solanum lycopersicum* L. cv 'Dwarf San Marzano'

The cultivated tomato is one of the most important economic crops in the world and is challenged by numerous pests that can damage its nutritional and market value (Lange & Bronson, 1981). The tomato plant has long been viewed as a model organism of the Solanaceae family and has therefore been a major research subject in both field and laboratory conditions (Bergougnoux, 2014). To date, several studies have investigated the interaction among different varieties of tomato plants – herbivores and beneficial microbes such as AMF (Lax et al., 2011; Song et al., 2013; Shafiei et al., 2022), EF (Panyasiri et al., 2007; Labbé et al., 2009; Shrivastava et al., 2015; Silva et al., 2020), PGPR (Shavit et al., 2013; Ling et al., 2022) and PGPF (Battaglia et al., 2013; Leonetti et al., 2017; Coppola et al., 2019; Lelio et al., 2021). In this thesis, a "dwarf" variety of the plant was selected in order to obtain a better standardized physiological response in a confined environment compared to commercial variety of 'San Marzano 2' (Coppola et al., 2019).

3.2.2. Sweet pepper, *Capsicum annuum* L. cv 'IDS RZ F1'

Sweet pepper**,** also known as bell pepper, is a perennial herbaceous and important vegetable crop within the Solanaceae family. *Capsicum annuum* has been studied by several research working in the field of multitrophic interaction among beneficial microbes and herbivores (Herman et al., 2008; Mantzoukas & Lagogiannis, 2019; Allegrucci et al., 2020; Pappas et al., 2021).

3.3.Stink bugs

3.3.1. The southern green stink bug, *Nezara viridula* (L.) (Hemiptera: Pentatomidae)

As a highly polyphagous pest of a wide variety of crops, the southern green stink bug causes significant damage to tomato, soybeans, potato, and several other crops (Panizzi et al., 2000). There is widespread distribution of *N. viridula* throughout tropical, subtropical and warm temperate regions of Eurasia, Africa, Australia and Americas (Todd, 1989; Musolin, 2010; Rabitsch, 2010). As a consequence of climate changes, the species is expanding its original range in Europe towards the north (Conti et al., 2021).

3.3.2. The brown marmorated stink bug, *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae)

The brown marmorated stink bug is an invasive pentatomid species native to East-Asia which has spread worldwide in recent years (Leskey & Nielsen, 2018). It is a polyphagous pest with approximately 300

host plants, including species of agricultural importance, several fruits, vegetables, row crops, nuts, and ornamental plants (Nielsen & Hamilton, 2009). The severe and widespread damage caused by this species has been documented in invaded areas such as the USA and Europe due to its reproductive capacity, feeding behavior and ability to fly long distances (Zhu et al., 2012; Bariselli et al., 2016; Kriticos et al., 2017). Several recent attempts have been conducted to manage this invasive species through chemical and biological control (Dieckhoff et al., 2017; Kuhar & Kamminga, 2017).

3.4. Egg parasitoids

3.4.1. *Trissolcus basalis* (Wollaston) (Hymenoptera: Scelionidae)

Trissolcus basalis is a solitary egg parasitoid species that attacks the eggs of several phytophagous pentatomid bugs. It is now present in many parts of the world and considered as an effective biological control agent employed successfully in classical biological programs against *N. viridula* (Colazza & Bin, 1995; Corrêa-Ferreira & Moscardi, 1996; Conti et al., 2021). *Trissolcus basalis* is demonstrated to locate its associated host by exploiting host-related cues and plant synomones (Colazza et al., 2010; Conti & Colazza, 2012; Guarino et al., 2017; Peri et al., 2018).

3.4.2. *Trissolcus japonicus* (Ashmead) (Hymenoptera: Scelionidae)

Trissolcus japonicus is the primary parasitoid of *H. halys* in Asia, where it is capable of achieving parasitism rates ranging from 50 to 90% (Zhang et al., 2017). To date, it has been fortuitously and intentionally introduced in many countries such as the United States (Talamas et al., 2015), Canada (Abram et al., 2019), Switzerland (Stahl et al., 2019), Italy (Peverieri et al., 2018) and Germany (Dieckhoff et al., 2021). *Trissolcus japonicus* has been expected to establish throughout Europe according to the bioclimatic modeling software CLIMEX (Avila & Charles, 2018). In Italy, the field release of T. *japonicus* was authorized for the first time in the summer of 2020 (Orrù et al., 2022).

4. Outline of the thesis

This thesis is based on the following chapters, which are specified in each section and summarized in Figure 2.

Chapter 1 provides insight into (i) the plant-mediated effects of plant-growth promoting fungi *T. harzianum* strain T22 on the performance of southern green stink bug *N. viridula* and (ii) the underlying molecular mechanism in tomato plants induced by *N. viridula* feeding. In this context, insect performance bioassays and plant defense-related gene expression were carried out. The results were discussed from a molecular perspective to demonstrate how *T. harzianum* T22 affects direct plant defenses.

Chapter 2 focuses on three different multitrophic systems consisting of (i) tomato plant – *T. harzianum* T22 – *N. viridula* – *T. basalis*; (ii) sweet pepper plant – *T. harzianum* T22 – *N. viridula* – *T. basalis*; (iii) sweet pepper plant – *B. bassiana* – *N. viridula* – *T. basalis*. Here the aim was to investigate the impact of two beneficial fungi on indirect plant defenses. This was done by investigating the olfactory behavioral responses of the egg parasitoid *T. basalis* toward the plants induced by *N. viridula*.

Chapter 3 investigates the behavioral and molecular basis of interactions between tomato plants – *T. harzianum* T22 – *H. halys* – *T. japonicus*. Insect performance bioassays, gene expression analysis and Y-tube olfactometer bioassays were conducted to determine whether *T. harzianum* T22 enhances direct and indirect plant defenses against *H. halys*.

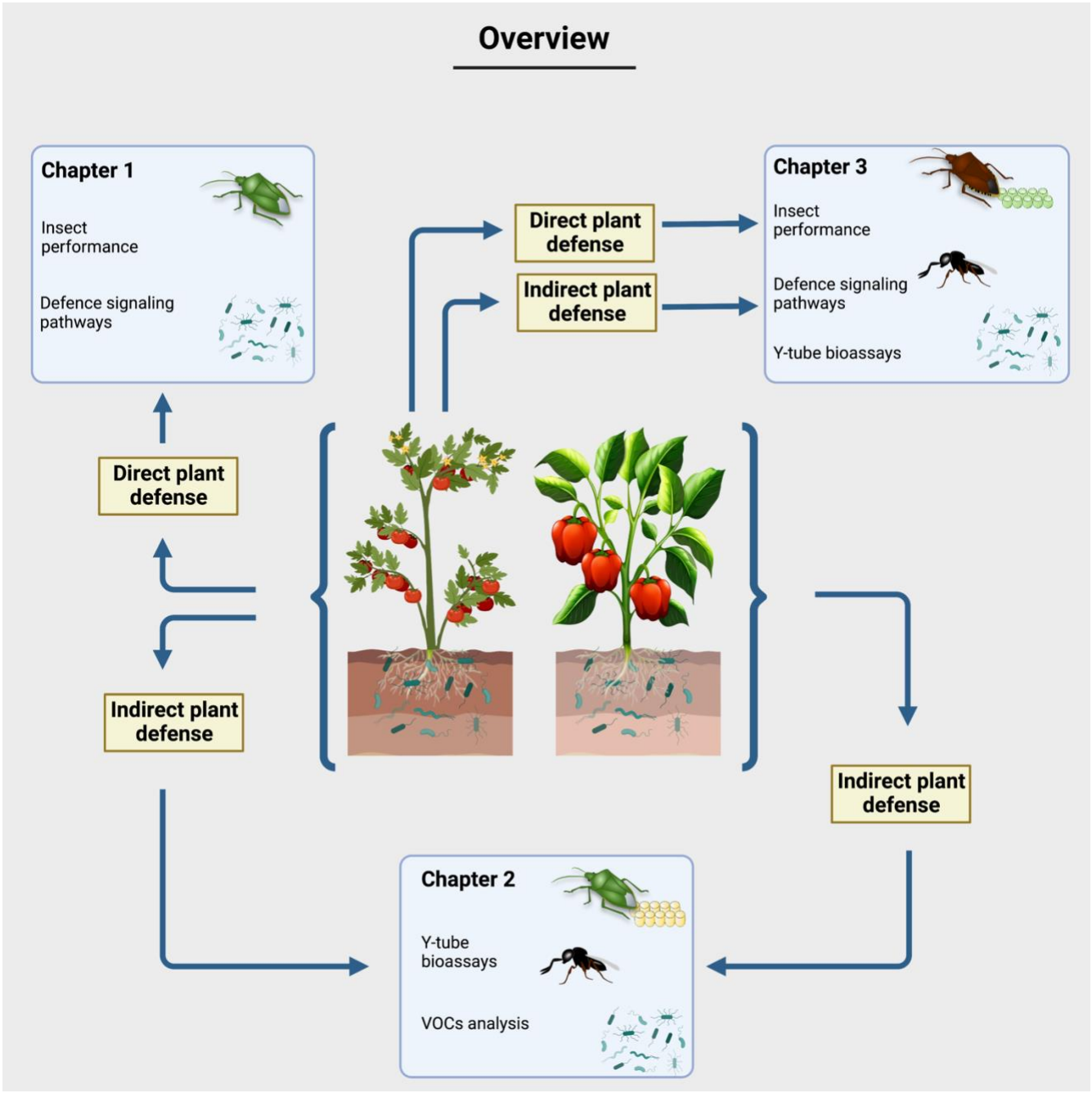

Fig. 2. Conceptual overview of the thesis

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Chapter 1

Trichoderma harzianum strain T22 modulates tomato plant direct defense in response to *Nezara viridula* feeding activity

Alınç, T., Cusumano, A., Peri, E., Torta, L., & Colazza, S. (2021). *Trichoderma harzianum* strain T22 modulates direct defense of tomato plants in response to *Nezara viridula* feeding activity. Journal of Chemical Ecology, 47, 455-462.

Abstract

Plant growth-promoting fungi belonging to genus *Trichoderma* are known to help plants when dealing with biotic stressors by enhancing plant defenses. While beneficial effects of *Trichoderma* spp. against plant pathogens have long been documented, fewer studies have investigated their effect on insect pests. Here, I studied the impact of *Trichoderma* root colonization on the plant defense responses against stink bug feeding attack. For this purpose, a model system consisting of tomato plant, *Solanum lycopersicum* cv 'Dwarf San Marzano', *Trichoderma harzianum* strain T22 and the southern green stink bug, *Nezara viridula*, was used. We firstly determined stink bug performance in terms of relative growth rate and survival on tomato plants inoculated by *T. harzianum* T22. Then, I evaluated relative expression of plant defense-related genes on inoculated plants induced by stink bug feeding. I found evidence that *T. harzianum* T22 affects tomato defense responses against *N. viridula* nymphs leading to reduction of growth rate. The results also showed that *T. harzianum* T22 enhances plant direct defenses by an early increase of transcript levels of jasmonic acid marker genes. Yet this effect was time-dependent and only detected 8 h after herbivore induction. Taken together, the findings presented here provide better understanding on the mechanisms underlying tomato induced resistance against herbivorous stink bugs.

Keywords: Stink bugs, Beneficial microbes, *Solanum lycopersicum*, Jasmonic acid signaling pathway, Pentatomidae

1. Introduction

Plants can mount direct and indirect defense strategies by activating their immune system after herbivorous insect attack (Walling, 2000). Direct defenses negatively affect the performance of herbivores feeding on plants via physical (e.g., trichomes, thorns and waxes) and/or chemical mechanisms (e.g., toxins and deterrents) (Howe & Schaller, 2008). Indirect defenses enhance the recruitment of natural enemies of the attacking herbivore via emission of herbivore-induced plant volatiles (Kessler & Baldwin, 2001; Rasmann et al., 2005; D'Alessandro & Turlings, 2006). It is increasingly recognized that plants are not alone when interacting with herbivorous insects as they have established, over evolutionary time, associations with microbial symbionts that could influence plant defenses against herbivory (Pineda et al., 2010; Sugio et al., 2015). In fact, several studies have indicated that plant defenses can be promoted by beneficial microbes, such as plant growth-promoting rhizobacteria (Kessler & Baldwin, 2001; Dicke & Hilker, 2003), mycorrhizal fungi (Pozo & Azcón-Aguilar, 2007), endophytic fungi (Stein et al., 2008) and plant growth-promoting fungi (Harman et al., 2004; Segarra et al., 2009).

Beneficial microbes have been used in agriculture due to their positive effects on plant survival, growth and yield through direct or plant-mediated mechanisms (Rodriguez & Sanders, 2015; Bender et al., 2016). However, these microbes do not only promote nutrient acquisition and improve tolerance to abiotic stresses, but they also lead to negative effects on aboveground and belowground biotic stressors (Guerrieri & Digilio, 2008; Pieterse et al., 2014). Among beneficial microbes, plant growth-promoting fungi (PGPFs) belonging to genus *Trichoderma* are well known as effective widespread biological control agents against plant pathogens (Harman et al., 2004; Woo et al., 2014). Whereas the defensive effect of *Trichoderma* spp. on plant pathogens is well- document (Hanson & Howell, 2004; Vinale et al., 2012), recent studies have also revealed their role in mediating plant defenses against insect herbivores, in particular against piercing-sucking pests. For example, phloem-feeding aphid survival was significantly reduced by tomato root colonization by *Trichoderma atroviride* strain P1 (Coppola et al., 2019a). Similarly, the performance of the cell-content feeder *Thrips tabaci* Lindeman (Thysanoptera: Thripidae) is negatively affected by onion plants colonized by *Trichoderma* spp. (Muvea et al., 2014). The population of the phloem-feeding insect *Trialeurodes vaporariorum* (Westwood) (Hemiptera: Aleyrodidae) was found to decline after inoculation with either *T. atroviride* MT-20, *T. atroviride* S-2 and *Fusarium oxysporum* (Fo162) (Menjivar et al., 2012).

Beneficial microbes affect plant defenses via alteration of signaling pathways that enhance plant gene expression and metabolism in order to inhibit development of biotic stressors (Verhagen et al., 2004; Pineda

et al., 2010; van de Mortel et al., 2012). In the regulation of plant defenses, phytohormones such as jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) play a key role against pathogens and herbivores (Pieterse & Dicke, 2007). JA is acquired from linolenic acid through octadecanoid pathway and triggers direct defenses to wounding, necrotrophic pathogens and herbivores, commonly chewing insects (Walling, 2000; Pieterse et al., 2012; Zhang et al., 2017). Chewing of plant parts by insects causes the deoxygenation of linolenic acid and activates an octadecanoid pathway that results in JA biosynthesis eventually leading to production of proteinase inhibitors, polyphenol oxidases, plant-specific toxins (e.g., glucosinolates, alkaloids, terpenoids) and attraction of insect parasitoids (Broadway & Duffey, 1986; Walling, 2000; War et al., 2012). However, defense-signaling pathways can be tailored depending on insect species adaptation to host plant or feeding mode (Stotz et al., 2000; Moran & Thompson, 2001). Thus, while JA signaling pathway is activated in response to mostly chewing herbivores, piercing-sucking insects induce mainly SA- related defenses (Walling, 2000). Recent studies have showed that *Trichoderma* spp. can induce systemic resistance by enhancing phytohormone signaling pathways and defense priming in plants (Conrath et al., 2015; Yuan et al., 2019). For example, root colonization by *T. atroviride* P1 induced a plant transcriptome reprogramming in which both SA and JA pathways were up-regulated (Coppola et al., 2019a). Yet another study showed that *T. harzianum* T78 limited the root knot nematode, *Meloidogyne incognita* (Kofoid and White) Chitwood (Tylenchida: Heteroderidae), infection cycle (i.e., galling, fecundity and root invasion) through priming the SA- and the JA-dependent path- ways in tomato roots (Martínez-Medina et al., 2017).

Stink bugs (Hemiptera: Pentatomidae) are considered a group of major pests in several crops which cause economically important yield losses worldwide (Conti et al., 2021). To date, limited evidence is available about direct defenses of plants against stink bug damage which suggests the involvement of both JA and SA signaling pathways. For example, both SA and JA signaling pathways in *Arabidopsis thaliana* (L.) plants are activated in response to *Eurydema oleracea* (L.) (Hemiptera: Pentatomidae) feeding (Ederli et al., 2020). A combination of oviposition and feeding activities by the brown marmorated stink bug, *Halyomorpha halys* Stål, (Hemiptera: Pentatomidae) induces the JA pathway in *Vicia faba* L. plants resulting in the activation of cysteine protein inhibitor genes and *NAI1* (Rondoni et al., 2018).

To the best of my knowledge, the role of beneficial microbes in enhancing plant defenses against stink bug feeding has not been investigated. Thus, unravelling the potential role of beneficial microbes against such piercing-sucking herbivores needs attention to provide a better insight on multitrophic interactions, which may help to develop new strategies for controlling these important pests. This was the aim of the study in which we investigated: (i) the performance of stink bug nymphs (both in terms of relative growth rate and

survival) on tomato plants after root inoculation by beneficial microbe; (ii) the underlying molecular mechanism by which beneficial microbe can affect pest performances. I explored the above-mentioned objectives using a multitrophic system consisting of the tomato plant, *Solanum lycopersicum* L., the PGPF *T. harzianum* T22 and the piercing-sucking herbivore *Nezara viridula* (L.). *Trichoderma harzianum* T22 is one of the *Trichoderma* strains that hold potential for sustainable crop production and is available as commercial product (Vitti et al., 2015) and *N. viridula* is a serious insect pest of tomato feeding on the leaves and fruits causing discoloration upon ripening and development of corky area below the fruit surface (Wakil et al., 2017).

2. Materials and methods

2.1. Fungal cultures, plant and insects

Trichoderma harzianum T22 was provided by University of Naples Federico II, Naples, Italy. The isolate was routinely grown and sub-cultured on potato dextrose agar (PDA Oxoid) under room conditions (Fig. 1). Spores were harvested from PDA plates by flooding with sterile distilled water and adjust- ed to 10^7 ml^{-1} spores/ml conidial suspension.

Fig. 1. The colony of *T. harzianum* sub-cultured on PDA.

Tomato (*S. lycopersicum*) cv 'Dwarf San Marzano' was used for all experiments. The plants were kept in a growth chamber following transplant procedures (see below for de- tails) and watered every other day. The growth chamber was set at 23 ± 2 °C 70 ± 5% RH and 14L:10D photoperiod condition and equipped with the light bulbs placed above the foliage providing a photosynthetic flux density of 600 mol photons m^{-2} s^{-1}. Each plant in the experiments was around 20 cm height with 3–4 fully expanded leaves.

The colony of *N. viridula* was reared in insect cages (47.5 × 47.5 × 47.5 cm) (Bug-Dorm-44,545, MegaView Science Co. Ltd., Taichung, Taiwan) under controlled conditions (24 ± 1 °C; 70 ± 5% RH and 14L:10D photoperiod). Insects were fed with fresh organic vegetables, sunflower and zucchini seeds. Water was provided as soaked cotton wool inside a 12-cm petri dish and paper towels were placed into

cages as oviposition substrates. Food was renewed every 2–3 days and newly laid eggs were collected on a daily basis to maintain the colony.

2.2. Fungal inoculation

Seed treatment of tomato was carried out as described by Coppola et al. (2019a) (Fig 2.). The surface of seeds was sterilized using 1% (*v*/v) sodium hypochlorite for 5 min and properly rinsed in sterile distilled water. The seeds were coated with a 10^7 sp. ml^{-1} conidial suspension of *T. harzianum* T22 or with water for control. Following air desiccation for 24 h, dried seeds were placed on water-moistened filter paper in a sterile petri dish kept in the dark at 25 °C. Germinated seedlings were the transferred in sterilized soil filled trays and maintained in a growth chamber at 23 ± 2 °C, 70 ± 5% RH and 14L:10D photoperiod condition. After 3 weeks, tomato seedlings were transplanted into 14-cm-diameter plastic pots.

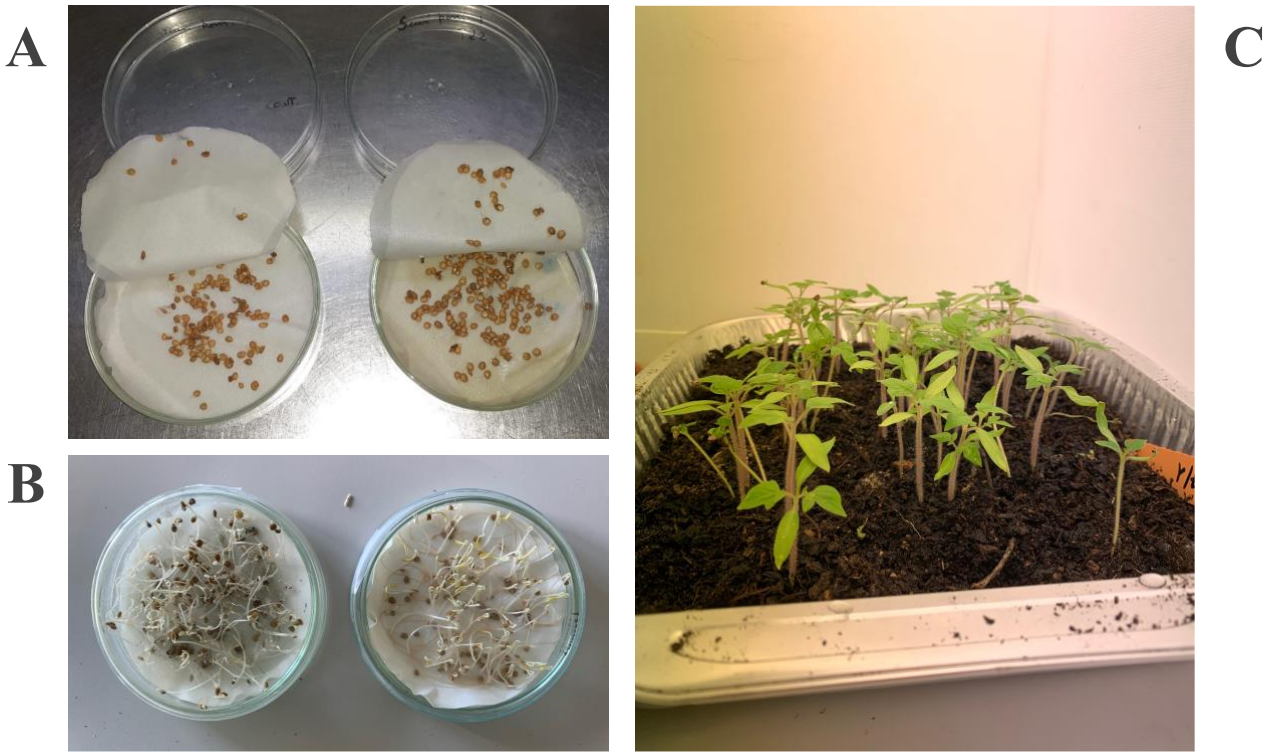

Fig. 2. Seed treatment procedure of tomato plants. The sterilized tomato seeds (A) were germinated on water-moistened filter paper in a petri dish (B) and transferred sterilized soil filled trays for seedlings stage (C).

2.3. Insect performance bioassays

Insect performance on tomato plants colonized by *T. harzianum* T22 was investigated by exposing each plant (inoculated or water control) to 3rd instar nymphs of *N. viridula*. Plants with

3–4 fully expanded leaves were enclosed together with 5 insects inside a nylon mesh bag (size = 30 cm × 40 cm; mesh count = 300 mesh/cm^2) (Fig. 2.). The nymphs were weighed on a Kern ABS-N analytical balance (Kern & Sohn, Germany) prior to bioassays and then allowed to feed on the plants for 1 week under controlled conditions (24 ± 1 °C; 70 ± 5% RH and 14L:10D photoperiod). After 1- week, nymphs were removed and re-weighed. To assess in- sect performance, we calculated for each plant, nymph mortality (i.e., % dead nymphs in relation to total nymphs) and nymph relative growth rate. To keep into account mortality effects on relative growth rate, we averaged the weight of the nymphs that were initially enclosed in the plant (i.e., initial average weight) as well as the weight of the nymphs that survived at the end of the experiment (i.e., final average weight). Thus, nymph relative grow rate was recorded as: (final average weight - initial average weight)/ initial average weight * 100). For each treatment, 15 replicates were carried out.

2.4. Plant induction, isolation of RNA and qPCR

To study tomato plant responses mediated by *T. harzianum* T22 against *N. viridula*, we quantified relative transcript levels of three defense marker genes involved in different signal- transduction pathways. As a marker of JA-signaling pathway we used *ToLOX D* whereas, as a marker of SA-signaling pathway, we used *ToPR-1*. We also investigated transcript levels of *ToPIN2*, a gene coding for a protein inhibitor.
Tomato plants were treated by exposing the youngest fully expanded leaf to a 4–5 d-old female of *N viridula*. Insects were individually confined on the leaf surface using a clip cage (3.8 cm diameter; 1 cm high) with a mesh-covered hole (3 cm diameter) and with the rim covered by a sponge ring to prevent damage to the leaf. The insects were allowed to feed for 8, 24 or 72 h, then the clip cages and the insects were removed, and leaf disks (3.8 cm in diameter) were excised from the treated leaf in order to be stored at −80 °C until gene expression analyses. Tomato plants were either inoculated with *T. harzianum* T22 at seed stage as described above and then induced by stink bug feeding (Treatment "T22Nv") or were only exposed to stink bug feeding (Treatment "Nv"). As control we used leaf disks collected from plants with empty clip-cages to assess gene expression level in the absence of herbivory (undamaged, non-inoculated plants). Five biological replicates, each consisting of a leaf disk per plant and per treatment, were performed. RNA was isolated using the ISOLATE II Plant RNA kit from Bioline according to the manufacturer's instructions. Two µg of total RNA was reverse-transcribed into cDNA using Bio-Rad's iSCRIPT cDNA synthesis kit in a 40 µL reaction volume according to the manufacturer's instructions. Primers (Table 1) were earlier designed (Coppola et al., 2015; De Palma et al., 2016). iQ SYBRGreen Supermix (Bio-Rad) was used to perform the real time qPCR reactions in

duplicate. The following PCR program was used for all PCR reactions: 95 °C for, 3 min followed by 40 cycles of 95 °C for 10 s, annealing temperature of 62 °C for 10 s and 72 °C for 30 s, with data collection at 72 °C. The PCR reactions were followed by a melt curve analysis to check for primer-dimer formation or unspecific PCR products. Relative changes in gene expression were assessed with the $2^{-\Delta\Delta Cq}$ method (Livak & Schmittgen, 2001). Delta-delta Cq values were calculated using the quantification cycle (Cq) values of the untreated plants and normalizing using the Cq values of the reference gene Actin.

Table 1. Specific primers for quantitative PCR of plant-defense related genes

Oligoname	Sequence	Name/Gene symbol	Primer from
LoxD Fw	TTCATGGCCGTGGTTGACA	lipoxigenase D (LOX D)	Coppola et at 2015
LoxD Rv	AACAATCTCTGCATCTCCGG		
PIN II Fw	CCAAAAAGGCCAAATGCTTG	Proteinase inhibitor II (PIN II)	Coppola et at 2015
PIN II Rv	TGTGCAACACGTGGTACATCC		
PR1 Fw	ATGCAACACTCTGGTGGACCTT	PR1	Coppola et at 2015
PR1 Rv	CCATTGCTTCTCATCAACCCA		
Actin-fw	CACCACTGCTGAACGGGAA	Actin	De Palma et al 2016
Actin-rev	GGAGCTGCTCCTGGCAGTTT		

2.5. Statistical analysis

Logistic regression with binomial error distribution and log- link function was used to test whether stink bug mortality was affected by the *T. harzianum* T22 inoculation treatment. ANOVA was used to test whether stink bug relative growth rate on tomato plants was affected by fungal inoculation treatment. ANOVA was also used to test if transcript levels of plant genes were significantly affected by the treatments and by different stink bug feeding times (8, 24 and 72 h). Gene transcription data were log-transformed before analyses to meet assumptions of normality and heteroscedasticity and model fit was assessed with residual plots. Post-hoc differences between the treatments were tested using Tukey tests. Data were analyzed with R statistical software (R Core Team, 2019).

3. Results

3.1. Effect of *Trichoderma harzianum* T22 on insect performance

Inoculation of tomato plants with *T. harzianum* T22 did not affect the mortality of *N. viridula* 3rd instar nymphs (GLM, $\chi^2 = 0.05$, df = 1, $P = 0.827$) (Fig. 3a). However, a significant effect of *T. harzianum* T22 was found in terms of relative growth rate (ANOVA, $F = 5.49$, df = 1,28 $P = 0.026$) as the weight of nymphs feeding on plants inoculated with the fungus was lower than nymphs feeding on non-inoculated plants (Fig. 3b).

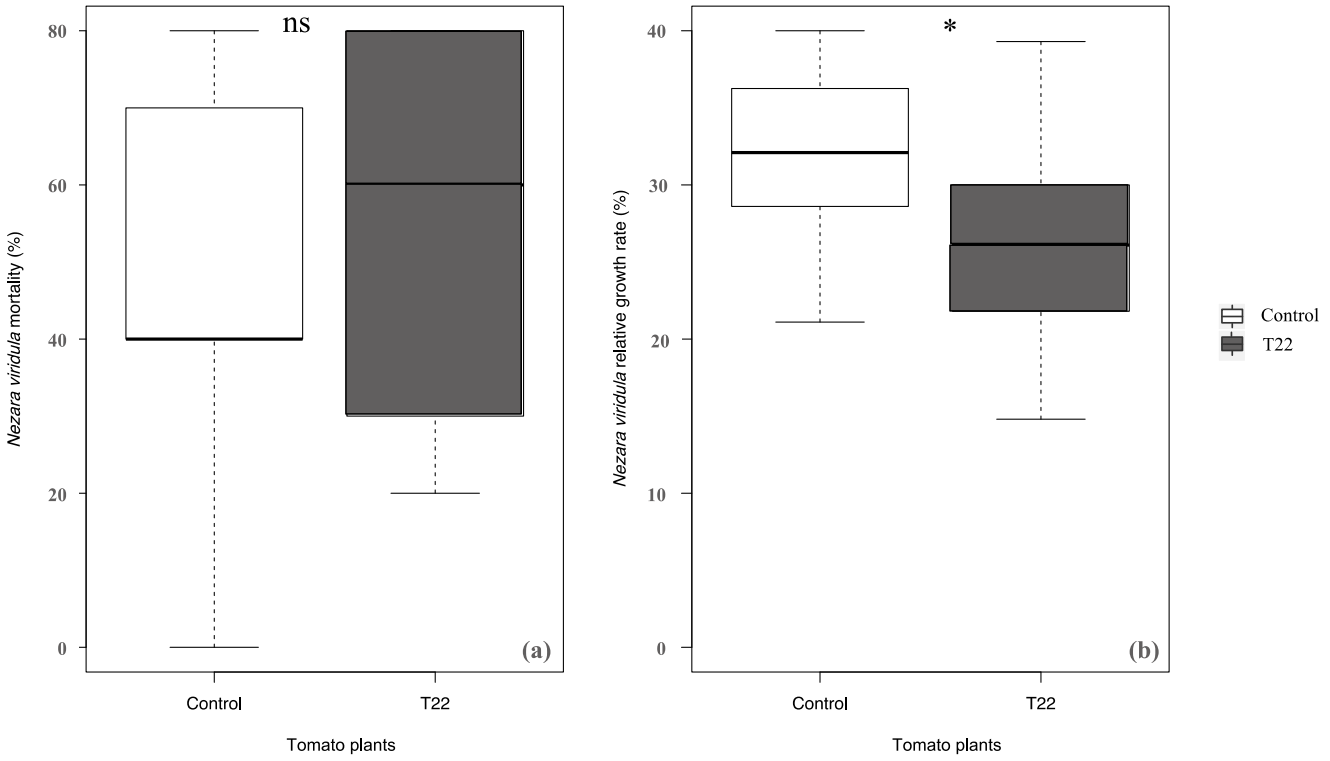

Fig. 3. Percentage of mortality (a) and relative growth rate (b) of *Nezara viridula* nymphs feeding on *Trichoderma harzianum* T22 inoculated (T22) versus non-inoculated (Control) tomato plants. Bold horizontal lines show medians, boxes contain the 25th–50th percentiles, whiskers show the upper and lower quartiles and points show outliers (ANOVA, $P < 0.05$, ns = no significant differences).

3.2. Effect of *Trichoderma harzianum* T22 on gene transcript levels

A significant effect of the treatment was found in transcript levels of *ToLOX D* as tomato plants treated with stink bug feeding were upregulated compared with undamaged control plants, regardless of the time point (ANOVA, 8 h: $F = 43.61$, df = 2,12 $P < 0.001$; 24 h: $F = 33.24$, df = 2,12 $P < 0.001$; 72 h, $F = 23.00$, df = 2,12 $P < 0.001$) (Fig. 4a). Yet the beneficial effect of the fungus was found only at 8 h time point with higher *ToLOX D* expression levels (treatment T22Nv compared with treatment Nv) (Fig. 4a). The transcript dynamics of *ToPIN2* were similar to *ToLOX D* as significant differences between plants exposed to stink bug feeding and un- damaged plants were found for each time interval (ANOVA, 8 h: $F = 10.33$, df = 2,12 $P = 0.002$; 24 h: $F = 21.07$, df = 2,12 $P < 0.001$; 72 h: $F = 5.95$, df = 2,12 $P = 0.016$). A significant upregulation of *ToPIN2* in the treatment T22Nv compared with Nv was found at 24 h (Fig. 4b). No significant differences in transcript levels of *ToPR1* were found between plants ex- posed to stink bug feeding and undamaged plants at 8 h and 24 h (ANOVA, 8 h: $F = 0.15$, df = 2,12 $P = 0.861$; 24 h: $F = 0.76$, df = 2,12 $P = 0.489$). A significant treatment effect was found at 72 h ($F = 5.32$, df = 2,12 $P = 0.022$) with non- inoculated plants exposed to *N. viridula* feeding showing higher transcript levels compared to undamaged plants (Fig. 4c). Transcript levels of *ToPR1* were overall similar across time intervals between non-inoculated and inoculated plants induced by stink bug feeding activity.

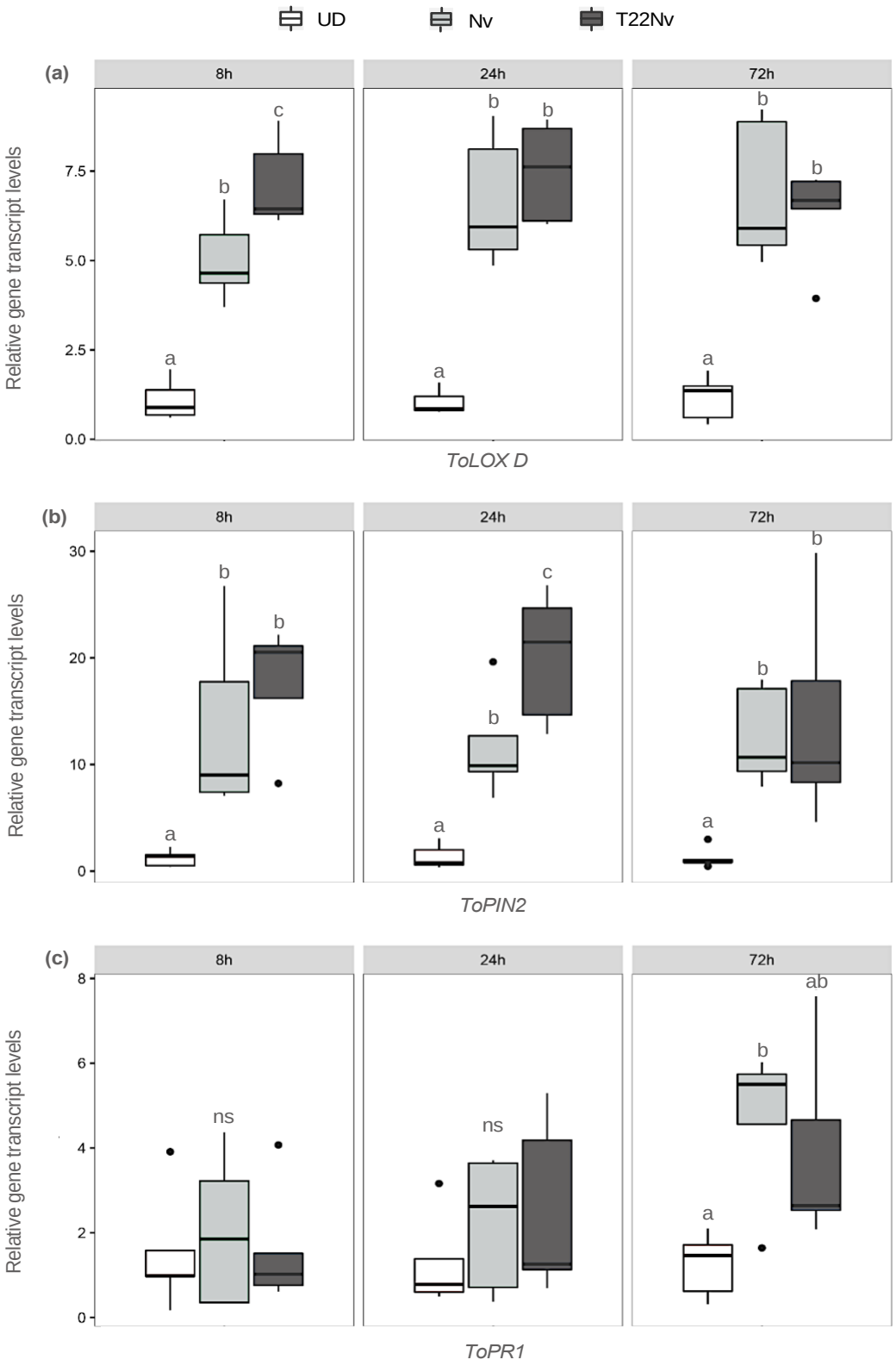

Fig. 4. Effects of tomato root colonization by *Trichoderma harzianum* T22 on relative expression of defense marker genes. Expression levels of the JA-marker gene *ToLOX D* (a), protein inhibitor gene *ToPIN2* (b) and SA-marker gene

ToPR1 (c) were analyzed in the leaves of tomato plants at 8h, 24h and 72h. UD = non-inoculated undamaged plants; Nv = non-inoculated plants damaged by *N. viridula* feeding; T22Nv = plants inoculated with *T. harzianum* T22 and subsequently damaged by *N. viridula* feeding. Bold horizontal lines show medians, boxes contain the 25^{th}–50^{th} percentiles, whiskers show the upper and lower quartiles and points show outliers. Different letters indicate statistically significant differences (ANOVA followed by Tukey *post hoc* test, $P < 0.05$, ns = no significant differences).

4. Discussion

This study provides the first evidence that root colonization of beneficial fungi affects direct defenses of plants in response to stink bug feeding activity. Although *N. viridula* mortality was not enhanced, we observed that the relative growth rate of nymphs was negatively affected when they fed on tomato plants previously inoculated with *T. harzianum* T22. To date, it has been shown that *Trichoderma* spp. colonization affects plant responses against herbivores which induce different feeding damage (Muvea et al., 2014; Contreras- Cornejo et al., 2018b). Plant responses to herbivory have often been linked to feeding patterns with a general dichotomy between piercing-sucking and chewing herbivores (Pieterse et al., 2009; Stam et al., 2014). Yet even within piercing-sucking insects, dissimilar feeding modes occur which could affect the way the plant responds to herbivores (Walling, 2000). For example, aphids can use their stylets to access to phloem content, whereas stink bugs can use their stylets to "lacerate and flush" plant tissues (Miles, 1972; Velikova et al., 2010). Yet *Trichoderma* spp. fungi have been shown to promote plant defenses against a wide range of insect attackers, regardless of the profound differences in feeding mode and wounding patterns induced by chewing and piercing-sucking insects (Menjivar et al., 2012; Muvea et al., 2014; Contreras-Cornejo et al., 2018a; Coppola et al., 2019a, b).

Few studies have investigated plant molecular responses against stink bug feeding even in the absence of beneficial microbes, thus, I first discuss the results showing how non- inoculated tomato plants respond to stink bug feeding and later I focus on how such responses are modulated by *Trichoderma* spp. At the molecular level, we show that tomato plants respond to *N. viridula* feeding by activating the JA- defense signaling pathway detected through an increase in expression levels of *ToLOX D* after 8 h of stink bug feeding. *ToPIN2* was also significantly upregulated already 8 h after herbivore feeding, probably as consequence of the activation of the JA-cascade. Considering that protein inhibitors are known to impair herbivore performance (Coppola et al., 2019a), upregulation of *ToPIN2* may play role in stink bug nymph reduced growth rate. Present results are in agreement with Peiffer and Felton, 2014 as they observed significant upregulation in *ToPIN2* expression in plants induced with salivary extract of the *H. halys*. Another study has also shown similar JA-mediated responses (i.e., activation of cysteine protein inhibitor gene and *NAI1*) in broad bean plants induced by feeding and oviposition activity by *H. halys* (Rondoni et al., 2018). Although gene expression patterns of tomato plants exposed to *N. viridula* feeding mainly induce activation of JA-induced defenses, we have evidence that SA-signaling pathway is also involved. In fact, the results demonstrate a significant increase of transcript levels of *ToPR1* after 72 h of *N. viridula* induction.

Involvement of SA-defense pathway is also documented for the stink bug *E. oleracea* feeding on *A. thaliana* plants (Ederli et al., 2020), although this study differed with the present results in the timing of plant responses to stink bug feeding, since early upregulation of *PR1* in *A. thaliana* plants occurred after 6 h of feeding. Interestingly, the same work found that transcript levels of the JA-dependent gene *AtPDF1.2* were induced later than *AtPR1*: taken together these studies provide evidence that both JA- and SA-defenses are involved in plant responses to stink bug feeding but there seems to be a specificity in terms of temporal patterns of molecular defenses (Ederli et al., 2020).

Concerning the role played by *T. harzianum* T22 at the plant- insect interface, this study indicated that root inoculation of tomato plants with *T. harzianum* T22 affected plant molecular responses to stink bug herbivory. Specifically, we found that inoculated plants significantly increase JA-defense signaling pathway as detected through an early increase (8 h) in *ToLOX D* and in *ToPIN2* (24 h) transcript levels compared with non- inoculated plants. Nonetheless, we did not find any evidence showing that *T. harzianum* T22 boosts SA-defense signaling pathway. In fact, expression levels of *ToPR1* were similar between inoculated and control plants, regardless of the time intervals investigated.

The early activation of JA-defense signaling pathway in plants inoculated with *T. harzianum* T22 indicates that these plants respond faster and more effectively to stink bug feeding. Thus, it is possible to hypothesize that the mechanisms by which *T. harzianum* T22 enhances resistance of tomato plants to *N. viridula* feeding could involve the so-called 'defense priming'. Priming is a phenomenon which sets the plants in 'alert' status ensuring faster and/or stronger defensive responses when attacks by biotic stressors occur (Van Der Ent et al., 2009; Lorito et al., 2010; Conrath et al., 2015; Martínez-Medina et al., 2017). To date, evidence showing that *Trichoderma* spp. prime plant defenses against biotic stressors is largely available for pathogens (Yedidia et al., 2003; Gallou et al., 2009; Perazzolli et al., 2011; Brotman et al., 2013). As in current study, a priming effect due to enhanced JA-defenses was induced by *T. harzianum* T78 in tomato plants challenged by the necrotrophic leaf pathogen *Botrytis cinerea* (Martínez- Medina et al., 2013). Furthermore, induction of a JA-related priming state by *T. harzianum* T22 in tomato plants has been observed against the aphid *Macrosiphum euphorbiae* (Thomas) (Hemiptera: Aphididae) (Coppola et al., 2019b).

To conclude, this study is the first piece of evidence showing that the strength of plant responses to stink bug feeding is positively affected by root inoculation with PGPFs as *Trichoderma*. Furthermore, we shed new light on the molecular mechanisms underlying the *Trichoderma*-induced resistance in tomato in response to feeding by *N. viridula*. Taken together, these results suggest that the use of beneficial

microbes to enhance plant defenses in a crop protection perspective appears a promising strategy to control an important group of pests such as herbivorous stink bugs in order to reduce pesticide application. Further works need to be carried out to establish whether beneficial microbes affect the indirect plant defenses against stink bugs and chemical communication in multitrophic interactions.

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Chapter 2

Role of beneficial fungi in mediating response of an egg parasitoid to oviposition-induced plant volatiles

In preparation

Part of this chapter was conducted in the Laboratory for Process Microbial Ecology and Bioinspirational Management, KU Leuven, Belgium under supervision of Prof. Bart Lievens.

Abstract

Plants can respond to egg deposition of insect herbivores by emitting plant volatiles that attract egg parasitoids. To date, research on trophic interactions has mainly considered chemical communication in tritrophic systems involving the plants, insect herbivores and associated egg parasitoids. Nonetheless, no information so far is available on how beneficial microbes modulates egg parasitoids' attractions toward plants induced by insect oviposition. To fill this gap, I evaluated the effects of colonization by two beneficial microbes, namely *Trichoderma harzianum* strain T22 and *Beauveria bassiana* ARSEF 3097 on tri-trophic systems consisting of tomato and sweet pepper plants, the southern green stink bug, *Nezara viridula* and an egg parasitoid, *Trissolcus basalis*. The results revealed that *T. harzianum* T22 enhanced the attraction of the egg parasitoid toward tomato and sweet pepper plants induced by *N. viridula* egg deposition. On the contrary, *T. basalis* females were found to be less attracted to the sweet pepper plants when the plants were first induced by endophytic colonization of *B. bassiana* and later by *N. viridula* egg deposition. Chemical analysis demonstrated that colonization by *T. harzianum* T22 on tomato plants resulted in changes in the composition of the blend of volatiles emitted by plants. These results could help to better understand the complex interactions occurring among plants, beneficial microbes, stink bugs and egg parasitoids.

Keywords: Plant–microbe–insect interactions, plant volatiles, *Trichoderma*, *Beauveria bassiana*, *Trissolcus basalis*

1. Introduction

For over 400 million years, insects and plants have coexisted in the same environment, serving as just two examples of the countless living organisms that interact with each other in a complex and ever-changing manner (Mello et al., 2002; Sugio et al., 2015). Plants are at the bottom of terrestrial trophic webs and interact with a variety of insects groups by establishing mutualistic or antagonistic interactions (Price, 2002). In a mutualistic interaction, both parties benefit from one another's. Examples of mutualisms include pollination, seed dispersion and plant protection. In antagonistic interactions, herbivores inflict damage to plants through feeding or laying eggs for their offspring to feed on host plant (Calatayud et al., 2018).

About half of one million insect species that are considered as pests, around 300.000 of these herbivorous species lay their eggs on plants (Schoonhoven et al., 2005). It is noteworthy that plants possess the ability to respond to insect oviposition and can thus defend themselves against insect attacks from the initial stage of egg deposition (Hilker & Fatouros, 2015). This mechanism underlying egg-induced plant defenses includes i) direct defense responses that target the insect eggs through wound tissue growth, desiccation, dropping and production of ovicidal substances; ii) "warning cue" responses that increase the plant defenses against herbivory which will occur soon); iii) indirect defense responses that change the chemical profile of epicuticular leaf waxes or emission of volatile organic compounds (VOCs) to recruit egg parasitoids) (Hilker & Meiners, 2010; Hilker & Fatouros, 2015; 2016). VOCs emitted in response to herbivore attack can be induced by feeding (the so-called herbivore-induced plant volatiles, HIPVs) or by egg deposition (i.e. oviposition-induced plant volatiles, OIPVs). HIPVs and OIPVs play a role in terrestrial food webs by mediating interactions among plants, herbivores and parasitoids (Pashalidou et al., 2015).

Plants are not alone when facing the threat of herbivory, as microbes have the ability to regulate plant's responses to herbivore attack. In the last years, there has been considerable interest in understanding plant – insect - microbe interactions that may have important ecological and evolutionary consequences for all involved species (Frago et al., 2012; Cusumano & Volkoff, 2021; French et al., 2021; Gruden et al., 2021). Overall, mutualistic symbiotic interactions between plants and microbes may lead to enhanced plant defenses through a regulation of induced systemic resistance (ISR) and/or plant systemic acquired resistance (SAR). To date, several studies have shown that beneficial microbes such as arbuscular

mycorrhizal fungi (AMF), endophytic fungi (EF), plant-growth promoting rhizobacteria (PGPR) and plant-growth promoting fungi (PGPF) can enhance direct plant defenses against herbivores (Gehring & Bennett, 2009; Pineda et al., 2010; Pozo et al., 2013). For instance, it has been found that the root colonization of the AMF *Glomus mosseae* reduced larval performance of a chewing caterpillar *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) (Song et al., 2013). Yet another study reported that root inoculation of *Arabidopsis* plants with *Pseudomonas fluorescens* WCS417r was found to reduce the larval weight of cabbage moth *Mamestra brassicae* L. (Lepidoptera: Noctuidae) (Pangesti et al., 2015). Furthermore, inoculation of *Trichoderma harzianum* strain T22 enhanced tomato defenses against the noctuid moth *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) and the aphid *Macrosiphum euphorbiae* (Thomas) (Hemiptera: Aphididae) (Coppola et al., 2019a).

Beneficial microbes may also affect indirect plant defenses and the recruitment of natural enemies as they are known to stimulate or change plant defense mechanisms which may impact the emission of HIPVs (Rasmann et al., 2017). To date, increasing evidence is showing that beneficial microbes can enhance the attraction of natural enemies in response to feeding herbivores (Gange et al., 2003; Contreras-Cornejo et al., 2018; Coppola et al., 2019b). Yet, no information up to now is available in order to understand how plant defenses induced by insect egg deposition can be modulated by beneficial microbes. This is surprising giving the fact that many insect herbivores lay eggs on the plant and thus plant responses to insect eggs are a ubiquitous first line of defense against herbivore attack (Fatouros et al., 2016).

To start filling this gap, I here investigated how colonization of two different species of beneficial fungi modulates indirect plants defenses induced by egg deposition of southern green stink bug, *Nezara viridula* (L.) (Hemiptera: Pentatomidae). For this purpose, I structured trophic systems including multiple study organisms; (1) plants: tomato, *Solanum lycopersicum* L., and sweet pepper, *Capsicum annuum* L.; (2) beneficial fungi: *T. harzianum* strain T22 and *B. bassiana* ARSEF 3097; (3) herbivores: *N. viridula* and (4) associated egg parasitoids: *Trissolcus basalis* (Wollaston) (Hymenoptera: Scelionidae). *Trichoderma harzianum* is a well-known effective biological control agent for controlling plant pathogens (Harman et al., 2004; Woo et al., 2014). Recent evidence suggests that this fungus can also be used in order to reduce plant damage inflicted by insect herbivores (Menjivar et al., 2012; Battaglia et al., 2013; Muvea et al., 2014; Coppola et al., 2019a). For instance, *T. harzianum* T22 affects

tomato direct defense responses against *N. viridula* nymphs leading to reduction of insect growth rate through an early increase of transcript levels of JA marker genes (See Chapter 1). Yet another beneficial fungus that I used in this thesis chapter, *B. bassiana*, is an entomopathogen fungus used to control herbivores by infecting their bodies (Mascarin & Jaronski, 2016). Furthermore, in the recent years increasing attention has been given to this entomopathogenic fungus due to its ability to act as a plant endophyte which might broaden the application of *B. bassiana* in biocontrol (McKinnon et al., 2017). For example, the survival of the cotton bollworm *Helicoverpa zea* (Boddie) (Lepidoptera; Noctuidae) larvae was reduced when they fed on cotton plants endophytically colonized by *B. bassiana* (Lopez & Sword, 2015). Nevertheless, how *B. bassiana* and *T. harzianum T22* affect the interactions among plants, herbivores, and egg parasitoids is still unclear. Hence, laboratory bioassays with tomato and sweet pepper plants were carried out in order to understand the role of beneficial fungi in mediating indirect plant defenses. In particular, 1) Y-tube experiments were employed to explore *T. basalis* olfactory responses to fungus-colonized plants attacked by the stink bug *Nezara viridula* and 2) GC-MS analysis of tomato plant headspace was carried out to link the egg parasitoid behavior with emission of induced plant volatiles.

2. Materials and methods

2.1. Fungal cultures, plants and insects

The colony of *T. harzianum* T22 was provided by the University of Naples Federico II in Naples (Italy). The isolate was routinely grown and sub-cultured at room temperature on potato dextrose agar (PDA; Oxoid).

Beauveria bassiana ARSEF 3097 was obtained from the Agricultural Research Service Collection of Entomopathogenic Fungal Cultures (ARSEF; New York, USA). It is originally isolated from a boll weevil cadaver in the Rio Grande Valley of Texas (Wright, 1996). The initial cultures were grown on a quarter strength plate (¼) of Sabouraud dextrose agar supplemented with yeast extract (Oxoid Holdings Ltd, United Kingdom) (SDAY). The fungal strains were then cultivated on SDAY for seven days at 25 °C in the dark.

Tomato (*Solanum lycopersicum* L.) cv 'Dwarf San Marzano' was used for *T. harzianum* T22 experiments. The plants were kept in a growth chamber following transplant procedures (see below for details) and watered every other day. The growth chamber was set at 23 ± 2 °C 70 ± 5% RH and 14L:10D photoperiod condition and equipped with the light bulbs placed above the foliage providing a photosynthetic flux density of 600 μmol photons m^{-2} s^{-1}. Each plant in the experiments was around 20 cm tall with 3–4 fully expanded leaves.

Sweet pepper (*Capsicum annuum* L.) cv 'IDS RZ F1' (Rijk Zwaan, De Lier, the Netherlands) were used for *B. bassiana* bioassays. Following transplanting operations, plants were cultivated in a 3:1 combination of potting mix (Universal potting mix; Agrofino, Ghent, Belgium) and perlite in a plant growth chamber adjusted to 23 ± 1 °C, 65 ± 2% RH, and 16L:8D photoperiod. The chamber was illuminated with 790 μmol photons m^{-2} s^{-1} photosynthetic flux density LED lamps. The plants were around 15 cm tall with 7-8 fully expanded leaves were employed for bioassays.

The colony of *N. viridula* was reared in separate insect cages (47.5 x 47.5 x 47.5 cm) (Bug-Dorm-44545, MegaView Science Co. Ltd., Taichung, Taiwan) under controlled conditions (24± 1°C; 70 ± 5% RH and 14L:10D photoperiod) and fed with fresh organic vegetables, sunflower seeds and broad bean (*Vicia faba* L.) plants. Water was served as soaked cotton wool inside a 12-cm diameter petri dish. Food was renewed every other day and egg masses were collected daily to maintain colonies of *N. viridula* and *T. basalis*.

Trissolcus basalis individuals were reared in 85-ml glass tubes under the same conditions for *N. viridula* and a drop of honey was provided every two days as a food source. In order to maintain the wasp colony, one of *N. viridula* egg mass was exposed to two females of *T. basalis* and parasitized egg masses were placed into separated glass tubes until the emergence of wasps. For all bioassays, 5-7 days old and naïve female wasps were individually isolated in small vials for 24 h and then transferred to the bioassay room to be acclimatized around 1 h before the tests.

2.2. Fungal inoculations

In order to obtain colonized tomato plants by *T. harzianum* T22, spores were harvested from PDA plates by flooding them with sterile distilled water and adjusting the concentration to 10^7 ml^{-1} of conidial suspension. The tomato seeds were treated as described by Coppola et al. 2019a. Briefly, the surface of the seeds was sterilized for 5 minutes with 1% (v/v) sodium hypochlorite and then rinsed in sterile distilled water. The seeds were coated with 10^7 spores per milliliter of *T. harzianum* T22 conidial suspension or with water as a control. Following 24 hours of air desiccation, dried seeds were germinated on water-moistened filter paper in a sterile Petri dish at 25°C in the dark. For emergence, germinated seedlings were transferred to sterile soil-filled trays and maintained at 20 ± 2°C and 14L:10D photoperiod in a growth chamber. Tomato seedlings were then transplanted into 14-cm-diameter plastic pots after three weeks.

Regarding the inoculation of sweet pepper, root dipping method for both beneficial fungi was used. The root of seedlings was first washed under running tap water and then immersed in a 10 mL solution of conidial spores. According to preliminary results, this inoculation approach consistently led to the establishment of the *B. bassiana* and *T. harzianum* T22 in sweet pepper. In addition, the roots of a second group of seedlings were immersed in 10 mL of physiological water to obtain non-inoculated (control) plants. The seedlings were then transplanted into plastic containers with a diameter of 10.5 cm and kept in the growth chamber for four weeks under the same environmental conditions as described earlier.

2.3. Plant treatments

The plants were individually exposed to a gravid *N. viridula* females for 24h in the rearing cages (47.5 x 47.5 x 47.5 cm) under controlled conditions (24 ± 2 °C; 70 ± 5% RH; 16 h:8 h L:D). After 24h, the stink bugs were removed from the cages and the treated plants were used for bioassays in the following day. The tomato and sweet pepper plants were differently treated as following:

(1) feeding (F): *N. viridula* females were allowed to feed on the non-inoculated plants but not to oviposit;

(2) feeding + oviposition (F_O): *N. viridula* females were allowed to feed and oviposit on the non-inoculated plants;

(3) feeding + *T. harzianum* T22 or *B. bassiana* (F_T22 and F_BB): the plants were first inoculated with related beneficial fungus and then exposed to *N. viridula* females to obtain only feeding punctures;

(4) feeding + oviposition + *T. harzianum* T22 or *B. bassiana* (F_O_T22 and F_O_BB): the plants were first inoculated with related beneficial fungi and then exposed to *N. viridula* females for feeding and oviposition;

(5) control plants inoculated with *T. harzianum* T22 or *B. bassiana* (T22 and BB): the plants were inoculated with related beneficial fungus but were not exposed to *N. viridula*;

(6) non-inoculated control plants (CTRL): healthy plants non-inoculated with *T. harzianum* T22 and uninfested by *N. viridula* females.

2.4. Y-tube bioassays

The following pairwise combinations were tested to evaluate the olfactory response of *T. basalis* to volatile compounds derived from tomato and sweet pepper plants that had been subjected to a variety of treatments in different study systems:

Tomato – *T. harzianum* T22 – *N. viridula* – *T. basalis* study system

1) F *vs* CTRL; 2) F_O *vs* CTRL; 3) T22 *vs* CTRL; 4) F_T22 *vs* T22; 5) F_O_T22 *vs* T22; 6) F_T22 *vs* F; 7) F_O_T22 *vs* F_O

Sweet pepper – *T. harzianum* T22 – *N. viridula* – *T. basalis* study system

1) F *vs* CTRL; 2) F_O *vs* CTRL; 3) F_T22 *vs* T22; 4) F_O_T22 *vs* T22

Sweet pepper – *B. bassiana* – *N. viridula* – *T. basalis* study system

1) F_BB *vs* BB; 2) F_O_BB *vs* BB

Y-tube olfactometer made from a polycarbonate body (stem 9 cm; arms 8 cm at 130" angle; ID 1.5 cm) sandwiched between two glass plates was employed in the bioassays (Fig. 1). A stream of medical-grade compressed air (approximately 80:20, N_2:O_2) coming straight from the cylinder, humidified by bubbling through a water jar, was regulated in each arm by a flowmeter at about 0.5 l min^{-1}. The device was illuminated from above by two 22-W cool white fluorescent tubes (full spectrum 5900 K, 11 W; Lival, Italy) and from below by an infrared source (homogeneous emission of wavelengths at 950 nm provided by 108 LEDs). Before entering the olfactometer arms, each air stream passed through a cylindrical glass cylinder (Ø = 12 cm: h = 52 cm) holding an odor source from a plant that had been treated. Wasp females were placed individually into the Y-tube olfactometer and after evaluating five parasitoid females, the stimuli were switched from their random assignment at the start of the bioassays. At each switch, the whole system was replaced with sanitized components. At the conclusion of the bioassays, the polycarbonate olfactometer and all glass components were cleaned using detergent and deionized water. The glass components were then baked at 180°C overnight. The behavior of wasps recorded for ten minutes using a monochrome CCD video camera (Sony SSC M370 CE) with a 12.5–75 mm/F 1.8 zoom lens. To exclude visible wavelengths, the camera lens was coated with an infrared pass filter (Kodak Wratten filter 87 A°). A video frame grabber (Studio PCTV–Pinnacle Systems, Mountain View, CA) was used to digitize analog video signals from the camera, which was then processed by XBug, a video tracking and motion analysis program (Colazza et al., 1999). Wasp response was measured in terms of residence time, i.e., the time spent by the wasps in each arm during the entire bioassay for 10 min in the dark room under controlled conditions. Each choice bioassay was replicated with 4 pairs and 10 female wasps per pairwise combination (about 40 wasps in total number).

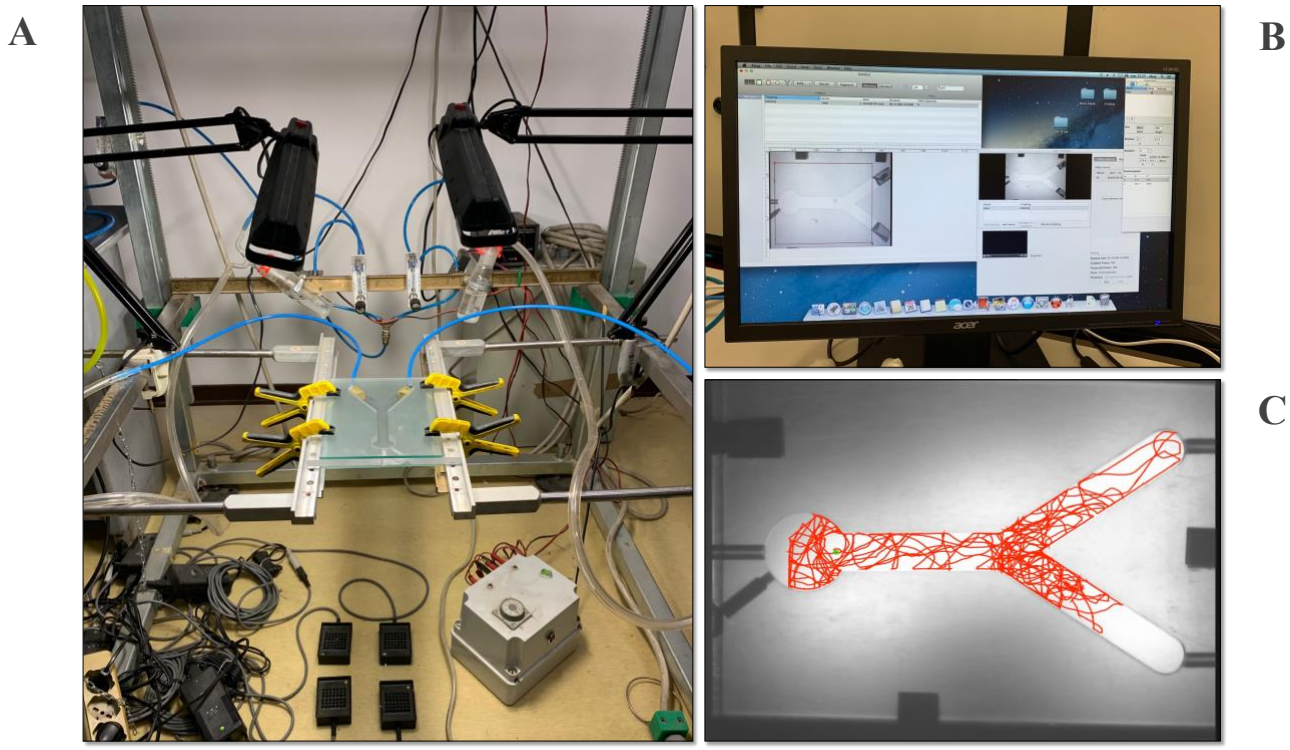

Fig. 1. Overview of experimental set-up during olfactometer bioassays. A) Y-tube system with air stream and illumination; B) A video tracking and motion analysis program, XBug; C) The motions of egg parasitoid female on Y-tube connected to odour source

2.5. Headspace collection and chemical analysis of VOCs

VOCs were collected from only the tomato plants used in *T. harzianum T22 – N. viridula – T. basalis* study system. Plants were prepared as described in the Y-tube bioassays with five replicates per treatment. For the headspace collections, plants were placed in a cylindrical glass chamber (inner Ø= 10 cm, h = 30 cm), VOC emissions by plants were collected using adsorbent traps, placed at the chamber outlet, made by glass tubes filled with PorapakQ (SigmaAldrich; 60 mg, 80–100 mesh) which were pre-cleaned with hexane and then heat conditioned for at least 2 h in a stream of nitrogen (100 mL/min). After 24 h traps were eluted with 400 µL of hexane, and the resulting extracts were stored at −20 ◦C in glass vials with Teflon cap liners until used for gas chromatography (GC) analysis.

Chemical analysis of the VOCs of the tomato plants was carried out through headspace dynamic air collection followed by gas chromatography and mass spectrometry (GC-MS). Analyses were performed by injecting 1 µL of extract on an Agilent 6890 GC system interfaced with an MS5973 quadruple mass

spectrometer equipped with a DB5-MS column in splitless mode. Injector and detector temperatures were 260 °C and 280 °C respectively. Helium was used as the carrier gas at a flow rate of 1.6 mL/min. The GC oven temperature was set at 40°C and then increased by 10°C/min to 250°C, with initial and final hold times of 5 and 20 min, respectively. Electron impact ionization spectra were obtained at 70 eV, recording mass spectra from 40 to 550 amu. Peak integration was carried out using ChemStation software.

VOC tentative identification was carried out using the NIST11 database. The retention index calculation, obtained by the injection of a 10-ppm solution of linear hydrocarbons C7-C30 and synthetic standards of α pinene, β myrcene, γ terpinene, limonene, β caryophyllene.

2.6. Statistical analysis

Data were analyzed by linear mixed models (LMMs) with the plant treatment as fixed factor and parasitoid nested within each plant pairs as random factor to account for pseudoreplication. For each pairwise combination, a separate LMM was used. Significance of the fixed term in the model was determined using likelihood ratio tests (LRTs). Model fit was assessed with residual plots.

For the volatile emission patterns, measured peak areas were first divided by the aboveground fresh mass of the plant. Then normalized peaks were analyzed through multivariate data analysis by non-metric multidimensional scaling (NMDS) using a Bray-Curtis distance matrix (Oksanen et al., 2013). Additionally, a permutational multivariate analysis of variance (perMANOVA) was carried out to test for significant differences in the chemical composition of the VOC blends, based on 1,000 permutations. We conducted the following pairwise permutation tests to stress on the VOC changes induced by fungal colonization of tomato plants: (1) CTRL vs T22, (2) F vs F_T22 (3) F_O vs F_O_T22). The analysis was performed using the adonis function from the Vegan package in R. All statistical analyses were carried out with R software, version 3.6.2 (R Core Team, 2019).

3. Results

3.1. Y-tube bioassays

Tomato – *T. harzianum T22 – N. viridula – T. basalis* study system

Trissolcus basalis females were significantly attracted to volatiles emitted by tomato plants exposed to *N. viridula* feeding and oviposition compared to control plants (F_O *vs* CTRL: χ^2=14.23, *df*=1, *P*=0.0001) (Fig. 2a). However, the wasps did not discriminate between volatiles emitted by feeding-induced plants and uninfested plants (F *vs* CTRL: χ^2=2.56, *df*=1, *P*=0.1094).

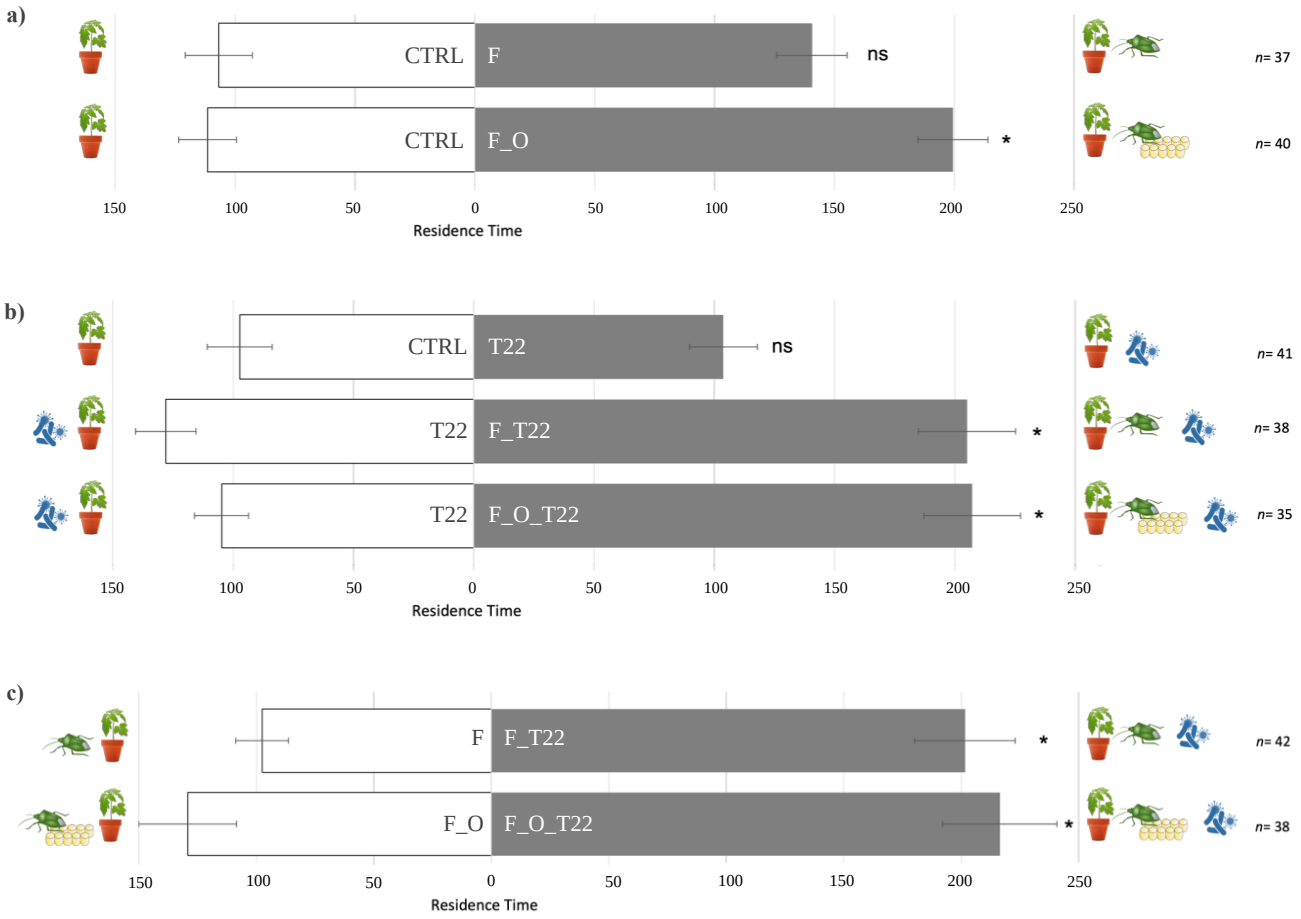

Fig. 2. Residence time (mean percentage ± SE) of *T. basalis* females in each arm of Y-tube connected to differently treated tomato plants. Treatments were non-inoculated and uninfested plants (CTRL); Trichoderma inoculated – uninfested plants (T22); non-inoculated – induced by *N. viridula* feeding plants (F); non-inoculated – induced by *N. viridula* feeding + oviposition (F_O) plants; *Trichoderma* inoculated – induced by *N. viridula* feeding plants (F_T22);

Trichoderma inoculated - induced by *N. viridula* feeding + oviposition plants (F_O_T22). (* asterisk indicates statistical significance; ns: not significant, *n* = number of replicates).

Trichoderma harzianum T22 inoculated tomato plants exposed to either *N. viridula* feeding or feeding + oviposition were found to be more attractive to female wasps than uninfested *T. harzianum* T22 inoculated plants (F_T22 *vs* T22: χ^2= 5.30, *df*=1 *P*=0.0212; F_O_T22 *vs* T22: χ^2=6.10, *df*=1, *P*=0.0134) (Fig. 2b). Yet the inoculation of *T. harzianum* T22 did not induce a significant response when inoculated and non-inoculated tomato plants were not infested by *N. viridula* females (T22 *vs* CTRL: χ^2=0.10, *df*=1, *P*=0.7528).

Trissolcus basalis female wasps exhibited significant preference for volatiles from *T. harzianum* T22 inoculated and infested tomato plants over infested but not inoculated ones (F_T22 *vs* F: χ^2= 5.48, *df*=1 *P*=0.0192; F_O_T22 *vs* F_O: χ^2=4.95, *df*=1, *P*=0.0260) (Fig. 2c).

Sweet pepper – *T. harzianum* T22 – *N. viridula* – *T. basalis* model system

Sweet pepper plants induced by *N. viridula* feeding + oviposition were more attractive to wasps compared with control sweet pepper plants (F_O *vs* CTRL, χ^2=8.38, *df*=1, *P*=0.003). Yet no significant differences in terms of wasp preferences were found between plants induced by *N. viridula* feeding and control plants (F *vs* CTRL: χ^2=0.02, *df*=1, *P*=0.8682) (Fig 3a)

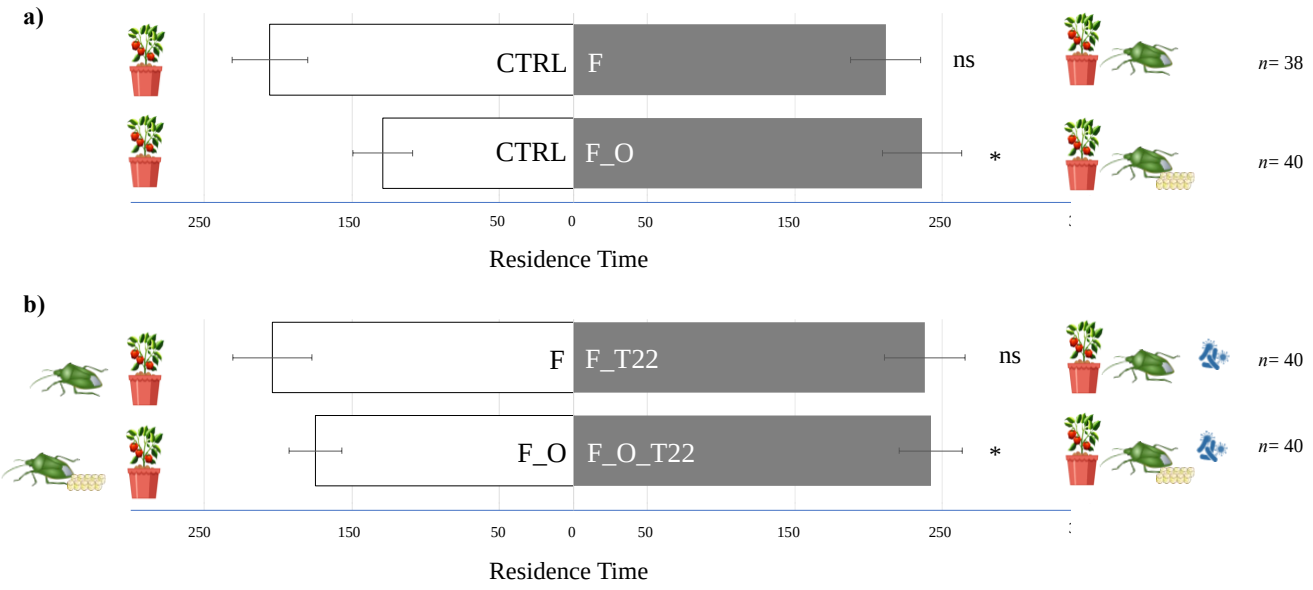

Fig. 3. Residence time (mean percentage ± SE) of *T. basalis* females in each arm of Y-tube connected to differently treated sweet pepper plants. Treatments were non-inoculated and uninfested plants (CTRL); non-inoculated – induced by *N. viridula* feeding plants (F); non-inoculated – induced by *N. viridula* feeding + oviposition plants (F_O); *Trichoderma* inoculated – induced by *N. viridula* feeding plants (F_T22); *Trichoderma* inoculated - induced by *N. viridula* feeding + oviposition plants (F_O_T22). (* asterisk indicates statistical significance; ns: not significant, *n* = number of replicates).

Females of *T. basalis* significantly preferred volatiles from *T. harzianum* T22 inoculated sweet pepper plants and induced by feeding + oviposition of *N. viridula* over non-inoculated but infested sweet pepper plants (F_O_T22 vs F_O: χ^2= 5.50, *df*=1 *P*=0.019) (Fig. 3b), while wasps did not show any significant attraction to sweet pepper plants when the inoculated and infested plants were compared to non-inoculated and infested plants (F_T22 vs F: χ^2=0.59, *df*=1, *P*=0.4396).

Sweet pepper – *B. bassiana* – *N. viridula* – *T. basalis* model system

The volatiles from *B. bassiana*-treated sweet pepper plants were less attractive to female wasps than those from non-inoculated plants when both plant pairs were induced by feeding + oviposition (F_O_BB vs F_O: χ^2= 6.85, *df*=1 *P*=0.008) (Fig. 4). However, the olfactory responses of wasps were

not significantly different in case of feeding + *B. bassiana* inoculated plants *versus* feeding induced plants (F_BB vs F: χ^2= 1.99, *df*=1 *P*=0.1583).

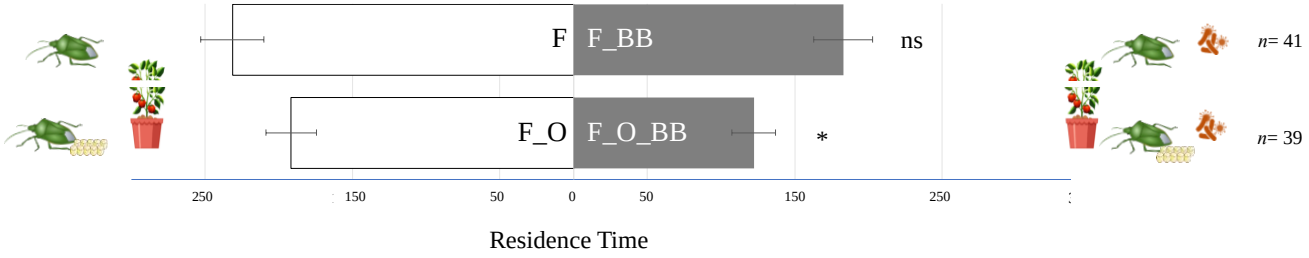

Fig. 4. Residence time (mean percentage ± SE) of *T. basalis* females in each arm of Y-tube connected to differently treated sweet pepper plants. Treatments were non-inoculated – induced by *N. viridula* feeding plants (F); non-inoculated – induced by *N. viridula* feeding + oviposition plants (F_O); *Beauveria* inoculated – induced by *N. viridula* feeding plants (F_BB); *Beauveria* inoculated - induced by *N. viridula* feeding + oviposition plants (F_O_BB). (* asterisk indicates statistical significance; ns: not significant, *n* = number of replicates).

3.2. Chemical analysis of VOCs

A total of twenty-one different volatile organic compounds was detected in the headspace of the differently treated tomato plants (Table 1). The compounds detected were thirteen monoterpene hydrocarbons, three sesquiterpene hydrocarbons, two green leaf volatiles, while two compound remained unidentified. Overall, differently treated plants emitted mainly the same compounds, but in different proportion. Qualitative differences were only found across F_T22 and T22 plants, as α-tujene were not detected from F_T22 and unk1 were not found in T22 plants. Significant differences due to root colonization by *T. harzianum* T22 were found between F_O and F_O_T22 plants (F = 9.55, df = 1, P = 0.024). (Fig. 5a). The greatest loadings of NMDS1, in descending order of absolute value, were found for (Z)-3-hepten-1-ol (0.637), hexanol (0.627), unknown 2 (0.575), tujene (0.501) and δ-elemene (0.496) whereas the greatest loadings of NMDS2 were for humulene (0.495), unknown 2 (0.245), unknown 1 (0.223) hexanol (0.177) and (Z)-3-hepten-1-ol (0.172). On the contrary, no significant differences were found between T22 and CTRL plants (F = 0.43, df = 1, P = 0.635). (Fig. 5b) or between F and F_T22 treated plants (F = 0.15, df = 1, P = 0.948) (Fig. 5c)

Table 1. Compounds detected in the headspace of the differently treated tomato plants: non-inoculated and uninfested plants (CTRL); non-inoculated – induced by *N. viridula* feeding plants (F); non-inoculated – induced by *N. viridula* feeding + oviposition plants (F_O); *Trichoderma* inoculated – induced by *N. viridula* feeding plants (F_T22); *Trichoderma* inoculated - induced by *N. viridula* feeding + oviposition plants (F_O_T22)

N	RT	LRI	chemical	group	CTRL	T22	FEED	F_T22	F_O	F_O_T22
ID_1	9.41	929	α-tujene	Mt. hd.	26.15 ± 12.15	5.65 ± 3.50	7.73 ± 3.17	0	0.78 ± 0.72	31.60 ± 18.20
ID_2	9.54	935	α-pinene*	Mt. hd.	2520.86 ± 911.21	1507.24 ± 473.03	1731.39 ± 448.29	1671.16 ± 531.76	410.87 ± 93.08	3202.03 ± 1025.90
ID_3	9.81	947	3 hexanol	GLV	7.97 ± 8.00	105.99 ± 78.43	12.38 ± 7.94	178.26 ± 145.14	171.87 ± 129.75	221.45 ± 111.34
ID_4	10.02	957	(Z)-3-hepten-1-ol	GLV	10.89 ± 8.64	130.87 ± 128.42	6.62 ± 4.20	214.92 ± 174.13	191.12 ± 149.49	259.81 ±131.95
ID_5	10.40	972	sabinene	Mt. hd.	1447.54 ± 713.26	576.86 ± 236.71	751.47 ± 173.16	896.21 ± 286.48	215.26 ± 62.68	1821.78 ± 747.87
ID_6	10.55	981	β-pinene	Mt. hd.	90.64 ± 37.65	118.94 ± 58.67	60.36 ± 14.21	46.18 ± 20.05	11.19 ± 3.30	125.56 ± 53.13
ID_7	10.76	990	β-myrcene*	Mt. hd.	73.89 ± 45.28	37.04 ± 9.21	47.69 ± 15.28	46.20 ± 13.98	20.03 ± 7.06	85.05 ± 30.61
ID_8	10.97	1000	2+ -carene	Mt. hd.	16859.32 ± 6821.54	7624.27 ± 3203.50	9801.90 ± 25.24	10271.88 ± 3439.79	2184.05 ± 515.57	15922.63 ± 3396.45
ID_9	11.12	1008	α-phellandrene	Mt. hd.	3361.39 ± 1632.64	2328.78 ± 934.06	1635.70 ± 506.53	1693.39 ± 610.78	335.42 ± 94.08	3359.81 ± 1166.80
ID_10	11.32	1019	α-terpinene	Mt. hd.	1039.55 ± 542.22	486.62 ± 152.95	391.58 ± 142.99	476.04 ± 186.34	83.62 ± 36.42	1134.65 ± 520.23
ID_11	11.47	1027	p-cymene	Mt. hd.	131.87 ± 37.90	127.45 ± 51.01	110.88 ± 23.99	88.60 ± 31.56	28.83 ± 10.12	97.58 ± 18.77
ID_12	11.56	1032	limonene*	Mt. hd.	8282.54 ± 4555.68	3122.434 ± 785.79	3506.28 ± 1045.31	3549.68 ± 1169.30	610.79 ± 280.67	3305.66 ± 741.80
ID_13	11.61	1035	β-phellandrene/cis	Mt. hd.	40111.58 ± 13702.34a	22185.38 ± 6901.66ab	21329.17 ± 5353.57ab	24933.60 ± 7934.46ab	6417.04 ± 1886.29b	41127.46 ± 8008.93a
ID_14	11.84	1047	o-cymene	Mt. hd.	140.52 ± 93.86	7.97 ± 4.38	6.23 ± 2.95	27.55 ± 14.55	9.67 ± 3.25	52.97 ± 43.65
ID_15	12.11	1062	γ-terpinene*	Mt. hd.	144.98 ± 67.47	58.79 ± 13.86	70.88 ± 19.03	45.04 ± 17.44	13.31 ± 3.73	136.65 ± 71.40
ID_16	12.58	1088	α-terpinolene	Mt. hd.	275.35 ± 139.40	81.24 ± 28.67	121.29 ± 33.16	85.34 ± 38.95	21.30 ± 6.72	268.95 ± 129.08
ID_17	13.11	1119	unkown 1		37.25 ± 19.97	0	8.46 ± 6.14	12.77 ± 6.14	1.50 ± 0.93	26.86 ± 10.67
ID_18	14.13	1181	unkown 2		27.51 ± 16.34	13.22 ± 5.42	2.63 ± 1.85	6.74 ± 3.80	0.50 ± 0.45	14.71 ± 8.96
ID_19	16.46	1340	δ-elemene	Sqt. hd.	30.86 ± 23.51	2.70 ± 1.82	6.40 ± 2.88	7.86 ± 4.20	1.12 ± 1.01	19.93 ± 14.10
ID_20	17.68	1432	trans-β-caryophyllene*	Sqt. hd.	272.56 ± 211.94	192.76 ± 53.39	59.4 ± 18.24	130.40 ± 50.10	31.11 ± 9.84	185.54 ± 88.53
ID_21	18.15	1468	humulene	Sqt. hd.	35.73 ± 30.96	23.00 ± 6.05	5.57 ± 2.32	11.84 ± 6.74	6.69 ± 2.91	21.69 ± 13.55

RT: retention time*; LRI*: linear retention index calculated using an n-alkane series (C7–C30) injected under the same conditions as samples.

* Compound confirmed by matching retention time and mass spectra with synthetic standards.

Groups: Mt., monoterpenes (C10); GLV., green leaf volatiles; Sqt., sesquiterpenes (C15); subgroups: hd. hydrocarbons

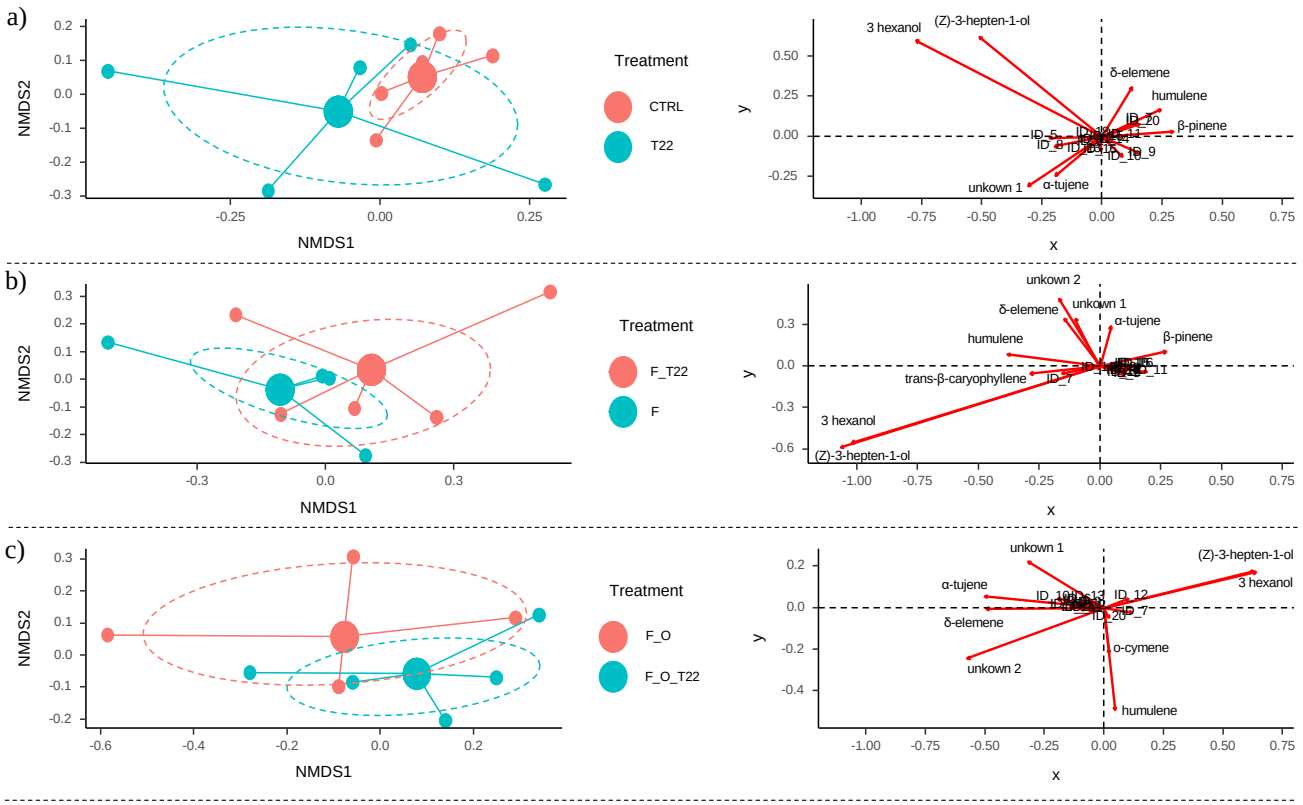

Fig. 5. Non-metric multidimensional scaling (NMDS) plot based on Bray–Curtis dissimilarities of relative amounts of chemical compounds found in differently treated tomato plants: non-inoculated and uninfested plants (CTRL); *Trichoderma* inoculated – uninfested plants (T22); non-inoculated – induced by *N. viridula* feeding plants (F); *Trichoderma* inoculated – induced by *N. viridula* feeding plants (F_T22); non-inoculated – induced by *N. viridula* feeding + oviposition (F_O) plants; *Trichoderma* inoculated - induced by *N. viridula* feeding + oviposition plants (F_O_T22). Each data point represents one sample. The distance between data points represents the degree of chemical dissimilarity between samples. The contribution of various classes of hydrocarbons is represented by arrows, whose direction is based on the amount of the respective class, and whose length represents the intensity of the correlation.

4. Discussion

To the best our knowledge, this is the first study to show how beneficial fungi affect indirect plant defenses induced by egg deposition. Since relatively no research has been carried out on tritrophic interaction among tomato - sweet pepper plants, *N. viridula* and *T. basalis,* I first focused on whether wasps can exploit the volatile compounds from plants induced by *N. viridula* in the absence of beneficial microbes. The olfactometer results demonstrated that *T. basalis* females had a stronger preference for volatiles from stink bug-infested plants than for volatiles from uninfested plants. This is in agreement with previous studies, which investigated *T. basalis* olfactory responses to OIPVs from different plant species induced by *N. viridula*. For example, Colazza et al. 2004 have shown that *T. basalis* females were attracted to combined feeding and oviposition activities of *N. viridula* on broad bean (*Vicia faba* L.) and french bean (*Phaseolus vulgaris* L.). By exploiting OIPVs, the egg parasitoids of stink bugs such as *T. basalis* are able to optimize their host foraging behavior (Conti & Colazza, 2012). Taken together, the results from leguminous and solanaceous plant system indicate that OIPV exploitation by *T. basalis* seems to be a widespread strategy in order to locate *N. viridula* eggs.

In the y-tube bioassays, *T. basalis* females were able to exploit volatile cues from tomato plants that have been inoculated by *T. harzianum T22*. However, the inoculation did not enhance the attraction of the parasitoid when the plants were not under attack from *N. viridula*. To date, the available information on how *Trichoderma* species enhance the attraction of parasitoids in the absence of their hosts suggests a specific effect of the *Trichoderma* species. For instance, *T. longibrachiatum* MK1 increased the attraction of *A. ervi* towards the uninfested tomato plants even in absence of aphid infestation (Battaglia et al., 2013), whereas the wasps did not exhibit any preference for uninfested plants colonized by *T. harzianum* T22 (Coppola et al., 2017). According to present results, concurrent stink bug infestation may be necessary to manifest changes in composition of VOCs to the benefit of associated natural enemies.

Present study systems clearly showed that inoculating tomato roots with *T. harzianum* T22, significantly enhanced the attraction of the egg parasitoid *T. basalis* towards tomato and sweet pepper plants infested by *N. viridula*. While there is currently a lack of knowledge on how *Trichoderma* mediates indirect plant defenses, the findings are in accordance with studies showing that inoculation of beneficial fungi results

in an increased attractiveness of host-infested plants to parasitoids (Battaglia et al., 2013; Parrilli et al., 2019; Coppola et al., 2019b). Remarkably, the same PGPF species used in this study, *T. harzianum* T22, has also been shown to regulate HIPVs emitted by aphid-infested tomato plants, resulting in an increased attraction of the aphid parasitoid *A. ervi* in comparison to non-inoculated plants (Coppola et al., 2017). Furthermore, the aphid parasitoid *A. ervi* is also attracted by aphid infested tomato plants colonized by *T. atroviride* P1 which correlated with qualitative changes in the HIPV blend due to z-3-hexenol and methyl salicylate (Coppola et al., 2019b).

In contrast to the outcome of *T. harzianum* T22, I observed that *B. bassiana*-colonized plants induced by *N. viridula* were less attractive to *T. basalis* in comparison with non-inoculated infested plants. This surprising result has also been reported for PGPR such as *Pseudomonas fluorescens* WCS417r, which reduces the attraction of the parasitoid *Diaeretiella rapae* (Hymenoptera: Braconidae) towards *M. persicae* infested plants when plant inoculation with *P. fluorescenens* occurred (Pineda et al., 2013). To the best our knowledge, there is no other available studies showing the same trend for sweet pepper plants colonized by *B. bassiana* up to now. Given that the interaction between plants colonized by beneficial microbes and insect herbivores is complex and species-dependent, future efforts should investigate whether *B. bassiana* changes the blend of induced plant volatiles that result in less attractive plants for *T. basalis*.

The GC-MS chemical analysis of the VOCs emitted from *T. harzianum* T22 inoculated tomato plants exposed to *N. viridula* feeding and oviposition evidenced differences in general composition of blend which are primarily determined by the higher presence of green leaf volatiles in fungus-inoculated plants. In addition, the quantity of α-tujene, humulene and δ-elemene were higher in the headspace of inoculated tomato plants compared to non-inoculated plants. Among the main chemicals identified that could play a role between the two treatments, δ-elemene was reported as being involved in tomato plant defense against herbivores (Kennedy, 2003). The amount of δ-elemene has been found higher in the headspace of tomato plants infested by the greenhouse whitefly *Trialeurodes vaporariorum* (Westwood) (Hemiptera: Aleyrodidae) and the South American tomato pinworm *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) (Anastasaki et al., 2015; Ayelo et al., 2021). Moreover, δ-elemene might be one of the important compounds for attraction of mirids predators (Hemiptera: Miridae) towards herbivore damaged tomato plants (Silva et al., 2018). The ability of parasitic wasps to orient toward such

semiochemicals has been found in specialists and general parasitoids species (Whitman and Eller, 1990; Meiners et al., 2002; Birkett et al., 2003; Shjojiri et al., 2006). Therefore, further research should be undertaken to reveal the role of specific compounds in the olfactory responses of *T. basalis*.

In conclusion, the current results suggest that inoculating tomato and sweet pepper plants with *T. harzianum* T22 enhance indirect plant defenses in response to *N. viridula* feeding and oviposition activity. Conversely, the attraction of *T. basalis* decreased in response to endophytic colonization of *B. bassiana* in sweet pepper plants resulting in a preference for non-inoculated plants. This study sheds light on the role of chemical communication among plant-insect-microbe interactions as a potential component of biocontrol strategies for the management of stink bugs. Nevertheless, the effect of *T. harzianum* T22 in indirect plant defenses needs to be investigated more closely to the natural condition. Moreover, further studies are required to reveal the interaction between *B. bassiana* and *T. basalis* through ecological and chemical approaches.

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Chapter 3

Beneficial fungal colonization of tomato plants enhances direct and indirect plant defenses against the stink bug *Halyomorpha halys*

In preparation

Abstract

The invasive brown marmorated stink bug *Halyomorpha halys* has become a serious economic pest species in several countries and it is often controlled with application of broad-spectrum insecticides. Due to the negative impacts of the chemical insecticides on the environment and non-target organisms, there is increasing demand to find alternative solutions to manage this pest in the invaded areas. I here investigated whether inoculation by the plant growth-promoting fungi *Trichoderma harzianum* strain T22 can enhance plant defenses against *H. halys*. I first determined how *T. harzianum* T22 modulates plant direct defenses against *H. halys* nymphs feeding on. The results showed that *T. harzianum* T22 reduced the relative growth rate of *H. halys* nymphs that developed on inoculated tomato plants. At the molecular level, *Trichoderma*-mediated effects were observed with higher transcript levels of the JA-dependent gene *PIN2* and SA-marker gene *ToPR1* in inoculated and infested by *H. halys* plants compared to non-inoculated – infested plants. Furthermore, I examined the effect of *T. harzianum* T22 on plant indirect defenses by investigating the behavior of the associated egg parasitoid *Trissolcus japonicus*. The results showed that *T. harzianum* T22 root colonization enhanced the attraction of *T. japonicus* females to volatiles from inoculated tomato plants induced by *H. halys* oviposition. This study suggests that *T. harzianum* T22 has a promising role in controlling *H. halys* by mediating direct and indirect defenses of tomato plants in multitrophic interactions.

Keywords: *Trichoderma harzianum*, PGPF, defense signaling pathways**,** stink bugs, *Trissolcus japonicus*

1. Introduction

The brown marmorated stink bug, *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae), has become an invasive pest of worldwide economic relevance. Because of its polyphagous feeding patterns, it can feed upon more than 300 species of annual and perennial plants (Lee et al., 2013; Bergmann et al., 2016), many of which are economically important, particularly within Fabaceae, Rosaceae, and Solanaceae (Yang et al., 2009; Rice et al., 2014). The presence of *H. halys* has already been documented in many states of the USA, Canada, and European countries such as Switzerland, France, Germany, Italy, Austria, Liechtenstein, Hungary, Serbia, Romania, Bulgaria and Greece (Haye et al., 2015; EPPO, 2022). Due to great dispersal abilities (Lee & Leskey, 2015), *Halyomorpha halys* can spread in flight to new areas and, in addition, it often hitches a ride on shipping crates and vehicles (Hoebeke & Carter, 2003). A combination of Europe's climate and suitable agricultural landscapes provides ideal conditions for the establishment and spread of this herbivore. Hence, it has been predicted that *H. halys* will likely spread throughout the European continent by causing great damage (Zhu et al., 2012; Wallner et al., 2014). For example, a localized outbreak of *H. halys* in Italy has led to 50% losses of fruit crops between 2014 and 2016 (Maistrello et al., 2017).

Currently, the primary method for controlling *H. halys* is still based on broad-spectrum insecticides although they have a negative impact on the environment, human health and non-target organisms such as natural enemies and pollinators (Nielsen et al., 2008; Leskey et al., 2012; Kuhar & Kamminga, 2017). Due to the absence of adequate pest control in certain circumstances and the significant environmental impact of insecticide treatments, other strategies for long-term management, such as biological control have received considerable attention (Abram et al., 2017). Given that native egg parasitoids have been demonstrated to be unsuccessful in controlling *H. halys* populations (Rice et al., 2014; Ogburn et al., 2016; Abram et al., 2017; Dieckhoff et al., 2017), the egg parasitoid *Trissolcus japonicus* (Ashmead) (Hymenoptera: Scelionidae) became a key biological control agents regarding its efficiency in original habitat of *H. halys* (Yang et al., 2009; Lee et al., 2013). Therefore, *T. japonicus* is recently being evaluated in *H. halys* invaded area such as USA and Europe (Milnes et al., 2016; Hedstrom et al., 2017; Sabbatini-Peverieri et al., 2020). Remarkably, following the detection of adventive populations of *T. japonicus* in Italy, its field release has been authorized in the summer of 2020 (Orrù et al., 2022).

It is worth noting that there are some challenges with introducing an exotic natural enemy as a candidate biological control agent (van Lenteren et al., 2006). On one hand, the release is regulated strictly by national and international rules, and on the other hand, the risk assessment process should be carefully

considered since it may have negative impacts on native ecosystem (e.g., interfering with native natural enemies, non-target organisms as evolutionary trap) (Thomas & Reid, 2007; Heimpel & Mills, 2017 Haye et al., 2020). It may take a considerable amount of time and effort to implement biological control agents from scratch. Therefore, there is increasing demand to find alternative solutions to manage this pest in the invaded areas.

In the recent years, in addition to their benefits to promote plant growth, nutrient uptake and increase plants defenses against phytopathogens, it has been shown that beneficial microbes can also help the plants against insect herbivore attacks (Guerrieri et al., 2004; Pineda et al., 2010; Rashid & Chung, 2017). For example, beneficial microbes can modify direct plant defenses and prime plants to respond faster and/or stronger to their insect stressors by the mechanism called induced systemic resistance (ISR) (Pieterse et al., 2014; Pineda et al., 2020). ISR is stimulated by beneficial microbes through the cascade of defense signaling pathways such as jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) (Shoresh et al., 2010). Moreover, it has been reported that indirect plant defenses can also be modulated by beneficial microbes by altering the blend of herbivore-induced plant volatiles (HIPVs) and oviposition-induced plant volatiles (OIPVs) which leads to increase on natural enemies' attraction (Rasmann et al., 2017; Francis et al., 2020). Taken together, beneficial microbes may be a promising alternative to chemical pest control by enhancing plant health in a multitrophic perspective.

Among beneficial microbes, plant growth-promoting fungi (PGPF) belonging to genus *Trichoderma* are well known to enhance plant growth and suppress the negative impact of plant pathogens (Harman et al., 2004, Woo et al., 2014). More recently, several studies have demonstrated that *Trichoderma* species also modulate plant defense systems against insect herbivores leading to reduced herbivory feeding, raised mortality rates of herbivores and increased attraction of natural enemies. (Woo et al., 2014; Poveda, 2021; see Chapter 1 and 2).

In this context, I hypothesized that *Trichoderma harzianum* strain T22 modulates both direct and indirect defenses of tomato plants against the stink bug *H. halys*, in line what observed in Chapter 1 and 2 with a different stink bug species. I here tested possible effects of the PGPF *T. harzianum* T22, in a multitrophic system consisting of tomato plants – *H. halys* and its associated egg parasitoid, *T. japonicus*. The aim of this chapter was to address the following questions: (i) can *T. harzianum* reduce the feeding activity of *H. halys*? (ii) if so, what is the underlying mechanism of this impact explained by molecular perspective? (iii) can *T. harzianum* enhance attraction of *T. japonicus* to OIPVs? The findings may provide important insights into understanding the role of PGPFs in plant responses against the invasive stink bug *H. halys*.

2. Materials and methods

2.1. Fungal cultures, plant and insects

The colony of *T. harzianum* strain T22 was provided by the University of Naples Federico II in Naples (Italy). The isolate was routinely grown and sub-cultured at room temperature on potato dextrose agar (PDA; Oxoid).

Tomato (*Solanum lycopersicum* cv 'Dwarf San Marzano') was used for all experiments. The plants were kept in a growth chamber following transplant procedures (see below for details) and watered every other day. The growth chamber was set at 23 ± 2 °C 70 ± 5% RH and 14L:10D photoperiod condition and equipped with the light bulbs placed above the foliage providing a photosynthetic flux density of 600 mol photons m^{-2} s^{-1}. Each plant in the experiments was around 20 cm height with 3–4 fully expanded leaves.

The colony of *H. halys* was established from field-collected individuals around Palermo, Italy, and reared in cages (47.5 × 47.5 × 47.5 cm; BugDorm-44545, Mega View Science Co., Ltd. Taichung, Taiwan) under controlled conditions (24 ± 2 °C; 70 ± 5% RH; 16L:8D photoperiod). The colony was fed with seasonal organic fresh vegetables and hazelnut seeds. Food was renewed every 2 days and water was provided with soaked cotton wool in small containers. Paper towels were hung inside each adult cage as oviposition substrates. Egg masses were collected daily and used to maintain both stink bugs and wasp colony.

The colony of *T. japonicus* was established from wasps emerging from *H. halys* sentinel egg masses located in fields around Turin, Italy and was reared on *H. halys* egg masses. Wasps were maintained in 85-ml glass tubes, fed with a honey–water solution (80:20 v/v) and kept in a controlled environmental room (24 ± 2°C; 70 ± 5% RH; 16L:8D photoperiod). *Halyomorpha halys* egg masses were singly glued onto a strip of paper and exposed to 2 female wasps for 24 h and, after emergence, male and female wasps were kept together to allow mating. Before the bioassays, 3–5 days old female and naïve wasps were individually isolated in a 2 mL vial with a drop of honey-water for 24 h and then transferred to the bioassay room in order to be acclimatized 1 h before the tests.

2.2. Fungal inoculation

Spores were harvested from PDA plates by flooding them with sterile distilled water and adjusting the concentration to 10^7 ml^{-} of conidial suspension. The tomato seeds were treated as described by Coppola et al. 2019a. Briefly, the surface of the seeds was sterilized for 5 minutes with 1% (v/v) sodium hypochlorite and then rinsed in sterile distilled water. The seeds were coated with 10^7 spores per milliliter of *T. harzianum* T22 conidial suspension or with water as a control. Following 24 hours of air desiccation, dried seeds were germinated on water-moistened filter paper in a sterile Petri dish at 25°C in the dark. For emergence, germinated seedlings were transferred to sterile soil-filled trays and maintained at 20 ± 2°C and 14L:10D photoperiod in a growth chamber. Tomato seedlings were then transplanted into 14-cm-diameter plastic pots after three weeks.

2.3. Insect performance bioassays

Insect performance on tomato plants colonized by *T. harzianum* T22 was investigated by exposing each plant (inoculated or water control) to 3rd instar nymphs of *H. halys*. Nymphs were weighed on a Kern ABS-N analytical balance (Kern & Sohn, Germany) prior to bioassays and then allowed to feed on the plants with 3–4 fully expanded leaves for 1 week under controlled conditions (24 ± 1 °C; 70 ± 5% RH and 14L:10D photoperiod). During bioassays, plants were enclosed together with 5 insects inside a nylon mesh bag (size = 30 cm × 40 cm; mesh count = 300 mesh/cm^2). After 1 week, nymphs were removed and re-weighed. To assess insect performance, I calculated for each plant, nymph mortality (i.e. % dead nymphs in relation to total nymphs) and nymph relative growth rate. I averaged the weight of nymphs that were initially enclosed in the plant (i.e. initial average weight) as well as the weight of the nymphs that survived at the end of the experiment (i.e. final average weight) in order to account for the mortality effect on relative growth rate. Thus, nymph relative grow rate was recorded as: (final average weight - initial average weight)/ initial average weight * 100. For each treatment, 10 replicates were carried out

2.4. Plant induction, isolation of RNA and qPCR

In order to analyze the effects of *T. harzianum* T22 on tomato plant responses to *H. halys*, I measured the relative transcript levels of three defensive marker genes implicated in several signal-transduction pathways. *ToLOX D* was observed as a marker for the JA-signaling route whereas *ToPR-1* was used for the SA-signaling pathway. Additionally, *ToPIN2*, a gene that codes for a protein inhibitor, was checked.

In order to treat tomato plants, the youngest fully expanded leaf was exposed to a 4- to 5-day-old female of *H. halys* (Fig 1.). Each insect was enclosed in a clip cage (4 cm in diameter and 1 cm in height) with a mesh-covered hole (3 cm in diameter) and its rim was covered with a sponge ring to prevent any damage to the leaf. The insects were allowed to feed for 8, 24 or 72 h, then the clip cages and the insects were removed, and leaf disks (3.8 cm in diameter) were excised from the treated leaf in order to be stored at −80 °C until gene expression analyses. Tomato plants were treated in two ways; (i) either inoculated with *T. harzianum* T22 at seed stage as described above and then induced by stink bug feeding (treatment "T22Hh") or were only exposed to stink bug feeding (treatment "Hh"). As control, I used leaf disks collected from plants with empty clip-cages to assess gene expression level in the absence of herbivory (uninfested and non-inoculated plants). Five biological replicates, each consisting of a leaf disk per plant and per treatment, were performed. RNA was isolated using the ISOLATE II Plant RNA kit from Bioline according to the manufacturer's instructions. Two µg of total RNA was reverse-transcribed into cDNA using Bio-Rad's iSCRIPT cDNA synthesis kit in a 40 µL reaction volume according to the manufacturer's instructions. Primers (Table 1) were earlier designed (Coppola et al., 2015; De Palma et al., 2016). iQ SYBRGreen Supermix (Bio-Rad) was used to perform the real time qPCR reactions in duplicate. The following PCR program was used for all PCR reactions: 95 °C for 3 min followed by 40 cycles of 95 °C for 10 s, annealing temperature of 62 °C for 10 s and 72 °C for 30 s, with data collection at 72 °C. The PCR reactions were followed by a melt curve analysis to check for primer-dimer formation or unspecific PCR products. Relative changes in gene expression were assessed with the $2^{-\Delta\Delta Cq}$ method (Livak & Schmittgen, 2001). Delta-delta Cq values were calculated using the quantification cycle (Cq) values of the untreated plants and normalizing using the Cq values of the reference gene Actin.

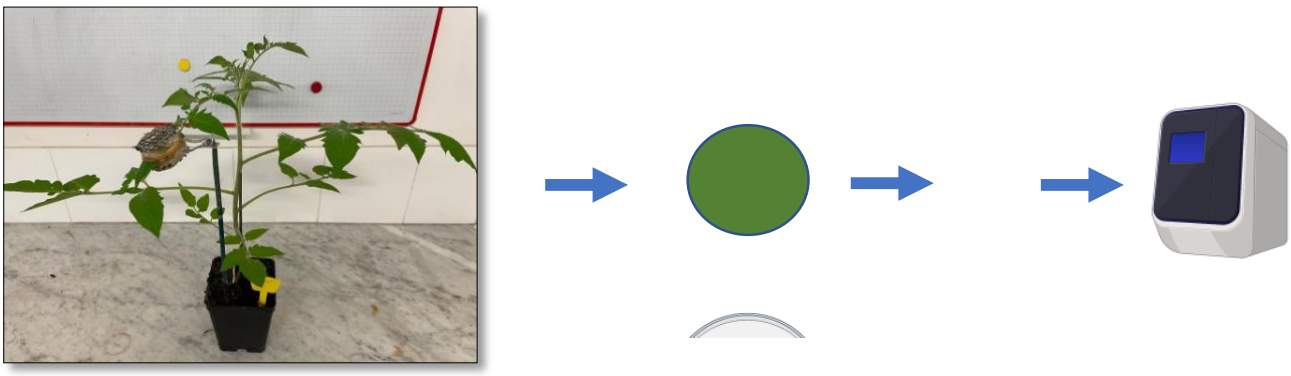

Fig. 1. The scheme of gene expression analysis

Table 1. Specific primers for quantitative PCR of plant-defense related genes

Oligoname	Sequence	Name/Gene symbol	Primer from
LoxD Fw	TTCATGGCCGTGGTTGACA	lipoxigenase D (LOX D)	Coppola et at 2015
LoxD Rv	AACAATCTCTGCATCTCCGG		
PIN II Fw	CCAAAAAGGCCAAATGCTTG	Proteinase inhibitor II (PIN II)	Coppola et at 2015
PIN II Rv	TGTGCAACACGTGGTACATCC		
PR1 Fw	ATGCAACACTCTGGTGGACCTT	PR1	Coppola et at 2015
PR1 Rv	CCATTGCTTCTCATCAACCCA		
Actin-fw	CACCACTGCTGAACGGGAA	Actin	De Palma et al 2016
Actin-rev	GGAGCTGCTCCTGGCAGTTT		

2.5. Y-tube bioassays

Plant treatments

Tomato plants was exposed to a gravid *H. halys* female for 24 hours in rearing cages (47.5 x 47.5 x 47.5 cm) under controlled conditions (24°C; 70% RH; 16h:8h L:D). This time allowed for the stink bugs to feed and oviposit on the plants. After 24h, the stink bugs were removed from the cages and the treated tomato plants were used for bioassays in the following day. Plants were discarded if after 24 hours no oviposition was observed on the leaves. Healthy plants placed into empty cages for same time interval were used as controls. The tomato plants were differently treated as following:

(1) feeding + oviposition (F_O): *H.halys* females were allowed to feed and oviposit on the non-inoculated plants;
(2) feeding + oviposition + *T. harzianum* T22 (F_O_T22): *H. halys* females were allowed to feed and oviposit on the PGPF inoculated plants;
(3) non-inoculated control plants (CTRL): non-inoculated plants kept in the same conditions without *H. halys* females.

Bioassays

The following pairwise combinations in Y-tube were tested to evaluate the olfactory response of *T. japonicus* to volatile compounds of tomato plants:

(i) feeding + oviposition *vs* control (F_O *vs* CTRL)

(ii) feeding + oviposition + T22 *vs* feeding + oviposition (F_O_T22 *vs* F_O)

A Y-tube olfactometer made from a polycarbonate body (stem 9 cm; arms 8 cm at 130" angle; ID 1.5 cm) sandwiched between two glass plates was employed in the bioassays. A stream of medical-grade compressed air (approximately 80:20, N_2:O_2) coming straight from the cylinder, humidified by bubbling through a water jar, was regulated in each arm by a flowmeter at about 0.5 l min^{-1}. The device was illuminated from above by two 22-W cool white fluorescent tubes (full spectrum 5900 K, 11 W; Lival, Italy) and from below by an infrared source (homogeneous emission of wavelengths at 950 nm provided by 108 LEDs). Before entering the olfactometer arms, each air stream passed through a cylindrical glass cylinder (Ø = 12 cm: h = 52 cm) containing a treated plant. Wasp females were placed individually into the Y-tube olfactometer and after evaluating five parasitoid females, the stimuli were switched from their random assignment at the start of the bioassays. At each switch, the whole system was replaced with sanitized components. At the conclusion of the bioassays, the polycarbonate olfactometer and all glass components were cleaned using detergent and deionized water. The glass components were then baked at 180°C overnight. The behavior of wasps was recorded for ten minutes using a monochrome CCD video camera (Sony SSC M370 CE) with a 12.5–75 mm/F 1.8 zoom lens. To exclude visible wavelengths, the camera lens was coated with an infrared pass filter (Kodak Wratten filter 87 A°). A video frame grabber (Studio PCTV–Pinnacle Systems, Mountain View, CA) was used to digitize analog video signals from the camera, which was then processed by XBug, a video tracking and motion analysis program (Colazza et al., 1999). Wasp response was measured in terms of residence time, i.e., the time spent by the wasps in each arm during the entire bioassay for 10 mins in the dark room under controlled conditions. Each choice bioassay was replicated with four 4 pairs and 10 female wasps per pairwise combination (about 40 wasps in total number).

2.6. Statistical analysis

Logistic regression with binomial error distribution and log- link function was used to test whether stink bug mortality was affected by the *T. harzianum* T22 inoculation treatment. ANOVA was used to test whether stink bug relative growth rate on tomato plants was affected by fungal inoculation treatment.

ANOVA was also used to test if transcript levels of plant genes were significantly affected by the treatments and by different stink bug feeding times (8, 24 and 72 h). Gene transcription data were log-transformed before analyses to meet assumptions of normality and heteroscedasticity and model fit was

assessed with residual plots. Post-hoc differences between the treatments were tested using Tukey tests. Data were analyzed with R statistical software (R Core Team, 2019).

3. Results

3.1. The effect of *T. harzianum* T22 on insect performance

Inoculation of tomato plants with *T. harzianum* T22 did not affect the mortality rate of 3rd instar nymphs of *H. halys* (GLM, $\chi 2$ =0.008, df=1, P =0.9273) (Fig. 2a). However, *H. halys* nymphs that developed on *T. harzianum* T22 inoculated tomato plants exhibited a lower weight compared to nymphs that developed on non-inoculated plants (ANOVA, F =5.84, df=1 P =0.046) (Fig. 2b).

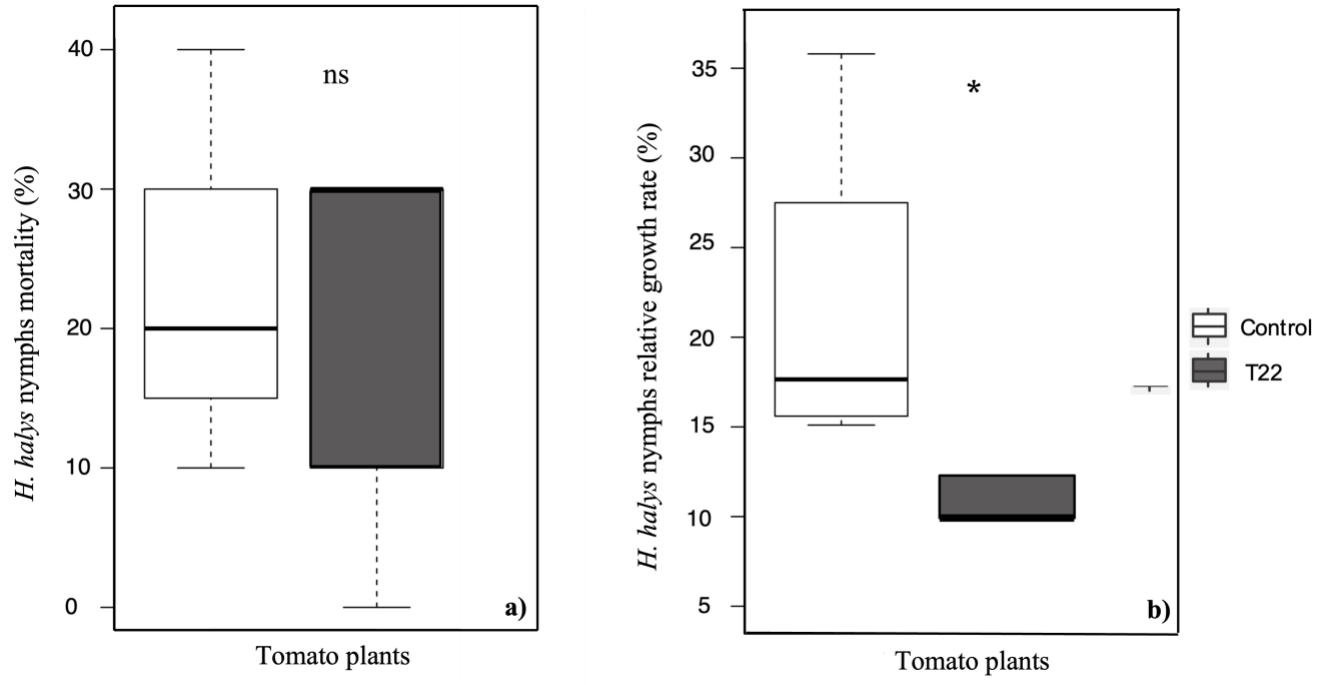

Fig. 2. Percentage of mortality (a) and relative growth rate (b) of *Halyomorpha halys* nymphs feeding on *Trichoderma harzianum* T22 inoculated (T22) versus non-inoculated (Control) tomato plants. Bold horizontal lines show medians, boxes contain the 25th–50th percentiles, whiskers show the upper and lower quartiles and points show outliers (ANOVA, $P < 0.05$, ns = no significant differences).

3.2. The effect of *T. harzianum* T22 on gene transcript levels

A significant upregulation of *ToLOX D* in tomato plants inoculated with *T. harzianum* T22 compared to control plants was found after 8h of *H. halys* feeding damage (ANOVA, $F = 2.5$, df = 4, $P = 0.066$) (Fig. 3a). However, there was no significant difference in transcript level of *ToLOX D* between inoculated and non-inoculated infested plant treatments (T22Hh and Hh) at the same time point (8h). The expression

levels of *ToLOX D* were overall similar at 24 and 72h between treatments. Feeding damage by *H. halys* resulted in transient activation of *PIN2* at 8h compared to control plants (ANOVA, $F = 5.95$, df = 4, $P = 0.001$) (Fig. 3b), yet the transcript level of *PIN2* declined after 24h. Remarkably, *T. harzianum* T22 significantly upregulated the expression of *PIN2* in T22Hh compared to Hh treatments along the tree time points. *Trichoderma*-mediated effects were also observed on the transcript levels of SA-marker gene *PR1* between inoculated and non-inoculated plants induced by *H. halys* feeding but only after 72h (ANOVA, $F = 1.4$, df = 4, $P = 0.26$) (Fig. 3c).

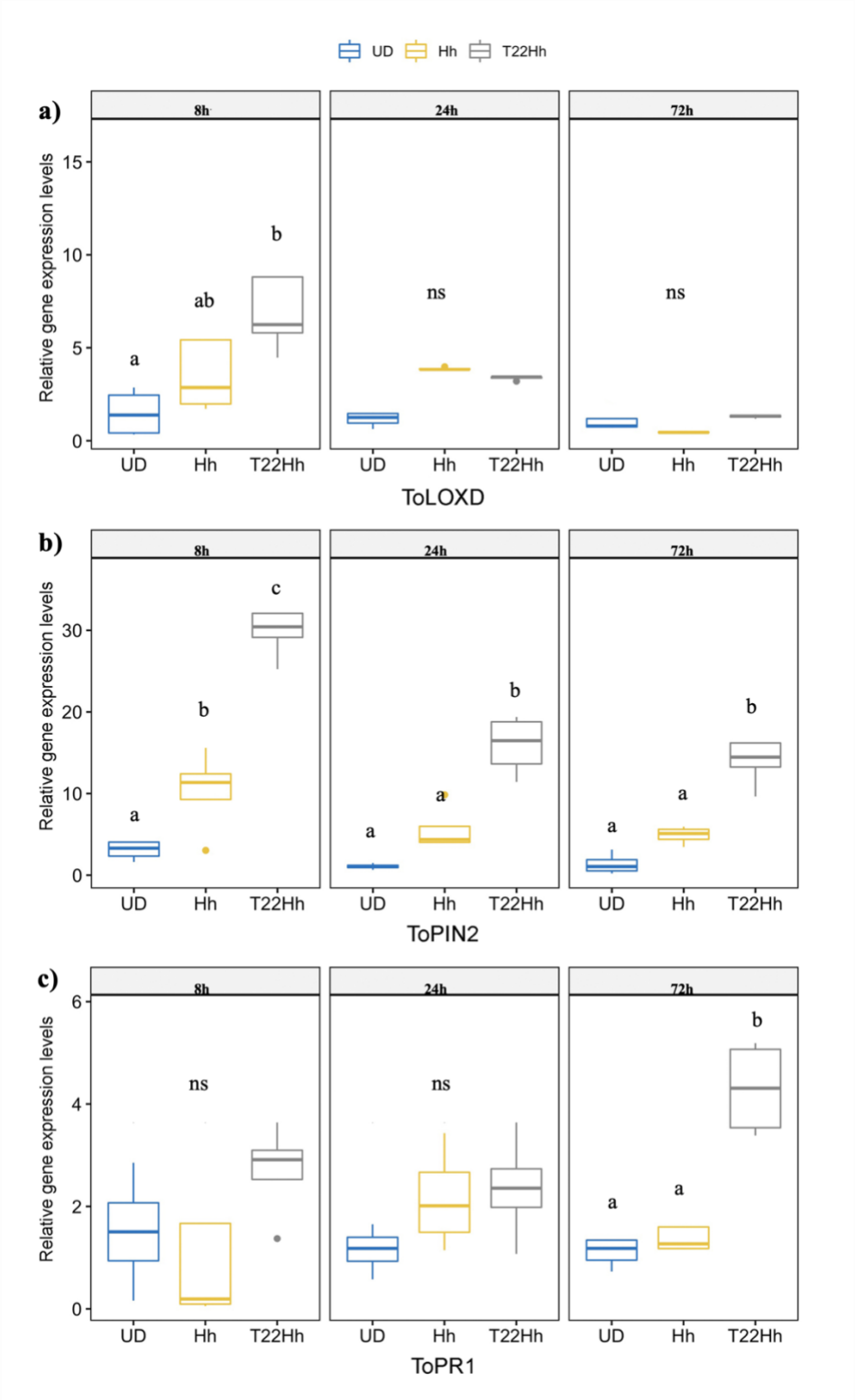

Fig. 3. Effects of tomato root colonization by *Trichoderma harzianum* T22 on relative expression of defense marker genes. Expression levels of the JA-marker gene *ToLOX D* (a), protein inhibitor gene *ToPIN2* (b) and SA-marker gene *ToPR1* (c) were analyzed in the leaves of tomato plants at 8h, 24h and 72h. UD = non-inoculated undamaged plants; Hh = non-inoculated

plants damaged by *H. halys* feeding; T22Hh = plants inoculated with *T. harzianum* T22 and subsequently damaged by *H. halys* feeding. Bold horizontal lines show medians, boxes contain the 25th–50th percentiles, whiskers show the upper and lower quartiles and points show outliers. Different letters indicate statistically significant differences (ANOVA followed by Tukey *post hoc* test, $P < 0.05$, ns = no significant differences).

3.3. Y-tube bioassays

Trissolcus japonicus females were significantly attracted to volatile compounds emitted by plants exposed to *H. halys* feeding and oviposition compared to control plants (F_O *vs* CTRL: χ^2=9.25, *df*=1, *P*=0.0023) (Fig. 4). Furthermore, root inoculation with *T. harzianum* T22 resulted in significantly higher attractiveness for *T. japonicus* to volatiles emitted by tomato plants exposed to feeding + oviposition compared to non-inoculated plants induced by *H. halys (*F_O_T22 *vs* F_O: χ^2=5.98, *df*=1, *P*= 0.0143).

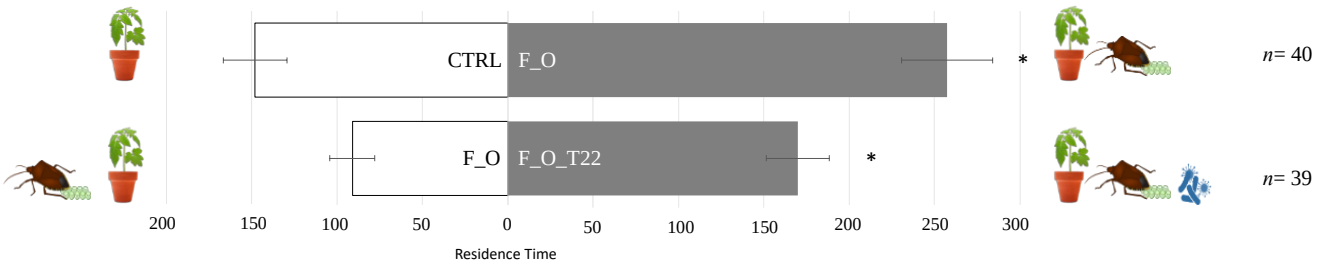

Fig. 4. Residence time (mean percentage ± SE) of *Trissolcus japonicus* females in each arm of Y-tube connected to differently treated tomato plants. Treatments were non-inoculated and uninfested plants (CTRL), non-inoculated – *H. halys* oviposited plants (F_O) and *Trichoderma* inoculated – *H. halys* oviposited plants (F_O_T22). Data were analyzed by means of GLMs (* asterisk indicates statistical significance; ns: not significant, *n* = number of replicates)

4. Discussion

Plants respond to herbivore attack by activating direct and indirect defense strategies (Karban & Baldwin, 1997). The direct defense impairs herbivore performance via physical (e.g., trichomes etc.) and chemical (e.g., toxins etc.) ways, whereas indirect defenses recruit natural enemies specific for herbivore via emission of induced plant volatiles such as HIPVs or OIPVs (Turlings et al. 1995; Dick & van Loon, 2000; Dicke and Baldwin 2010; McCormick et al., 2012; Turlings & Erb, 2018). This present study provides first comprehensive results indicating that inoculation by *T. harzianum* T22 in tomato plant enhances both defense strategies in response to *H. halys* infestation. I found that (i) colonization of *T. harzianum* T22 mediates direct defense of tomato plant by reducing the relative growth rate of *H. halys* nymphs; (ii) inoculation by *T. harzianum* T22 in tomato plants enhances defense signaling pathways after *H. halys* feeding; (iii) *T. harzianum* T22 enhances indirect defense of tomato plant by increasing the attraction of *T. japonicus* egg parasitoid when the plants are induced by *H. halys* oviposition. The results are in accordance with previous studies that investigated the role played by *Trichoderma* species in plant defenses (Battaglia et al., 2013; Contreras-Cornejo et al., 2018, b; Parrilli et al., 2019). For example, *Trichoderma atroviride* P1 was found to alter both direct and indirect defenses of tomato plant against the aphid *Macrosiphum euphorbiae* (Thomas) (Hemiptera: Aphididae) (Coppola et al., 2019a). In their study, Coppola et al. 2019a showed that *T. atroviride* P1 regulated the genes involved in the oxidative burst reaction which correlated with a reduction of the aphid longevity. Moreover, colonization by *T. atroviride* P1 leaded to a significant increase in the attraction of aphid parasitoid, *Aphidius ervi* Haliday (Hymenoptera, Braconidae).

Plant-herbivore interactions are affected by a variety of biotic and abiotic factors that influence plant nutrition, defense chemistry, feeding and oviposition of insects (Fox, 1981; Takabayashi et al., 1994; Gutschick, 1999). Among the biotic factors, the present study highlighted the importance of *T. harzianum* which negatively affected the growth rate of *H. halys* nymphs. The same outcome was found in the model system consisting of tomato plants, *T. harzianum* T22 and the stink bug species *Nezara viridula* (L.) suggesting that the observed effect might be a generalized responses mediated by the beneficial fungus against herbivore stink bugs (See Chapter 1). Indeed, in correlation of two studies, *T. harzianum* strain T22 did not affect the mortality of stink bug nymphs. In the light of these outcomes, *T. harzianum* strain T22 has a more efficient impact on feeding activities of stink bugs rather than such survival.

The JA- and SA- signaling pathways are dominant regulators of defense responses to herbivorous insects in plants (Glazebrook, 2005; Howe & Jander, 2008; Pieterse et al., 2009). Previous works have shown

that the beneficial effect of *Trichoderma* species against herbivores is strictly dependent on the relative abundance of the both antagonistic JA and SA defense pathways in tomato plants (Contreras- Cornejo et al., 2018; Coppola et al., 2019b; Jafarbeigi et al., 2020; Agbessenou et al., 2022). In the present study, I found that SA defense pathway was upregulated after 72h of *H. halys* infestation on tomato plants inoculated with *T. harzianum* T22 since I found increasing transcript levels of the marker gene *ToPR1*. Yet I could not detect any significant upregulation of *ToLOX D* in tomato plants inoculated with *T. harzianum* T22 across the time points of the study. However, tomato plants also responded to *H. halys* feeding by an increase in expression levels of JA-related gene, *ToPIN2*, after 8 h of stink bug feeding compared to control. Remarkably, for the same time interval, *H. halys* feeding induced a stronger expression of *ToPIN2* when the tomato plants were inoculated with *T. harzianum T22*. This trend suggests that *T. harzianum* T22 enhance direct defense of tomato plants against *H. halys* feeding by enhancing the expression level of *ToPIN2* which is JA dependent. Taken together, I have evidence showing that both JA and SA defense signaling pathways are involved in defense system against *H. halys*.

Several studies carried out on tomato have shown an induction of *PIN2* due to herbivory (Peiffer et al., 2009; Kawazu et al., 2012; Tian et al., 2012). For instance, Peiffer & Felton, (2014) reported that sheath extracts from *H. halys* elicited significant *PIN2* expression. Furthermore, it has been shown that tomato plants inoculated with *T. harzianum* T-78 accumulated higher levels of *PIN2* when attacked by the root knot nematode *Meloidogyne incognita* (Kofoid & White) Chitwood *(*Martínez-Medina et al., 2017). It is well known that, in response to herbivore damage, *PIN2* proteins are activated both locally and systemically following expression in leaves and contribute to resistance to herbivores which suffer a reduced growth and development (Pena-Cortes et al., 1988; Farmer & Ryan 1990; Lorito et al., 1994). Thus, upregulation of *ToPIN2* shown in this study may be responsible for lower relative growth rate of *H. halys* nymphs feeding on tomato plants.

Beneficial microbes can influence the composition of VOCs which eventually affect indirect plant defenses by recruiting natural enemies of the attacking herbivores (Kim & Felton, 2013; Stenberg et al., 2015; Van der Ent et al., 2009; Van Wees et al., 2008). For instance, a study by Coppola et al., 2017 found a significant increase in the attraction of the aphid parasitoid *A. ervi* as a result of colonization of *T. harzianum* T22. They linked this attraction to higher amount of z-3-hexenol, methyl salicylate and β-caryophyllene compounds in the headspace of colonized tomato plants under aphid attacks. Regarding the effect of *T. harzianum* T22 on the blend of VOCs compared to non-inoculated plants, the chemical

analysis of VOCs from *H. halys* and *T. harzianum* T22 induced tomato plants is needed to be addressed in future studies to provide greater insights into the role of PGPF in indirect plant defenses against *H. halys*.

In conclusion, these results suggest that inoculation of tomato plants with *T. harzianum* T22 enhances both direct and indirect defenses of tomato plants in response to attack by the invasive stink bug *H. halys*. This study reveals the role of chemical and molecular mechanisms among plant-insect-microbe interactions and may pave the way towards the development of biocontrol strategies for the management of stink bugs. However, it will be necessary to perform field studies in order to validate the plant-mediated effects of *T. harzianum* T22 under more realistic ecological conditions.

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Conclusion

Plants in natural and cultivated environments interact with various beneficial and detrimental organisms, including beneficial microbes and insects. To date, most of these interactions have been studied in two trophic levels (i.e., interactions between plants and beneficial microbes or between plants and arthropods). However, complex interactions occur in nature, as plants concurrently interact with both beneficial microbes and insect species. Understanding the complexity of plant-based trophic webs will contribute to the knowledge of how beneficial microbes mediate interactions between plants and insects in multitrophic systems.

The research presented here is an important first step in revealing the potential use of beneficial fungi to help the plants challenged by herbivore stink bugs. Results show that *T. harzianum* T22 enhances direct plant defenses against stink bugs feedings from ecological and molecular perspectives. Furthermore, *T. harzianum* T22 can also alter indirect plant defenses and increase the attraction of egg parasitoids associated with stink bugs. Remarkably, these beneficial effects were observed in diverse trophic systems, including different plants, stink bugs and egg parasitoid species. However, the interaction with a beneficial fungus does not always result in a benefit to the plant. Indeed, while these outcomes clearly demonstrated that *T. harzianum* T22 enhances plant defenses in response to herbivory, endophytic colonization of the beneficial microbe *B. bassiana* was found to decrease the attraction of egg parasitoid toward infested plants. Therefore, it is indeed noteworthy that the interactions between plants, beneficial microbes, and insects can lead to a complex interplay of outcomes requiring careful examination.

To conclude, evidence in this thesis shows that using beneficial microbes to enhance plant defenses in a crop protection perspective, plays a promising role in controlling an important group of pests such as herbivorous stink bugs. Nevertheless, how laboratory findings translate to field applications is still unclear, especially regarding the tritrophic interactions among plants with stink bugs and their natural enemies. To better understand the effectiveness of beneficial microbes, future studies must attempt to fill this knowledge gap in order to contribute to more environmentally friendly control strategies that rely less on chemical-based insecticides.

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