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The Impact of two Root-Symbiotic Fungi on Tomato Plant Indirect Defense Against *Spodoptera exigua* Herbivory

Master's Thesis

to gain the academic grade as a
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List of Abbreviations

ABA	Abscisic acid
AMF	Arbuscular mycorrhizal fungi
ANOVA	Analysis of variance
AOS	Allene oxide synthase
CFU	Colony-forming unit
ET	Ethylene
GA	Gibberellin
GC-MS	Gas chromatography-mass spectrometry
GLV	Green leaf volatiles
HIPV	Herbivore-induced plant volatile
IAA	Indole-3-acetic acid
IPM	Integrated pest management
ISR	Induced systematic resistance
JA	Jasmonic acid
LOX	Lipoxygenase
MeSA	Methyl salicylate
NIST	National Institute of Standards and Technology
PAL	Phenylalanine ammonia lyase
PCA	Principal component analysis
RT-qPCR	Reverse transcription quantitative real-time polymerase chain reaction
SA	Salicylic acid
SAMT	Salicylic acid carboxyl methyltransferase
SlEF	*Solanum lycopersicum* Elongation factor
TD-GC-MS	Thermal desorption-gas chromatography-mass spectrometry
TPS	Terpene synthase gene
PDA	Potato dextrose agar
PDMS	Polydimethylsiloxane
RH	Relative humidity
VOC	Volatile organic compound
ZR	Zeatin riboside

1. Introduction

Advanced knowledge on crop plant-pest interactions is more of interest for sustainable agricultural practices and the reduced usage of pesticides and fertilizer. The overuse of chemical pesticides and more hazardous pesticides leads to a severe decrease in biodiversity by threatening insects and organisms at higher trophic levels (Lechenet et al., 2017). The excessive use of nitrogenous and phosphorous fertilizers makes this phenomenon worse by contaminating soil and water bodies. Consequently, biodiversity loss and hampered ecosystem services make it difficult to secure our food resources and increase economic losses (OECD, 2019). To bring back nature to our agricultural land, the EU Biodiversity strategy for 2030 requires reducing the risk and using chemical pesticides by 50% and more hazardous pesticides by 50% (European Commission, 2020).

The Integrated Pest Management(IPM) concept recently emerged as a practical approach with excellent prospects to this demand (Stenberg, 2017; Fig. 1). IPM refers to careful consideration of all available plant protection methods and appropriate integrations of measures to the extent of "economically and ecologically justified levels," which is obliged to all EU member states to establish and implement in the European Union Directive (EC, 2009). Now IPM concept is

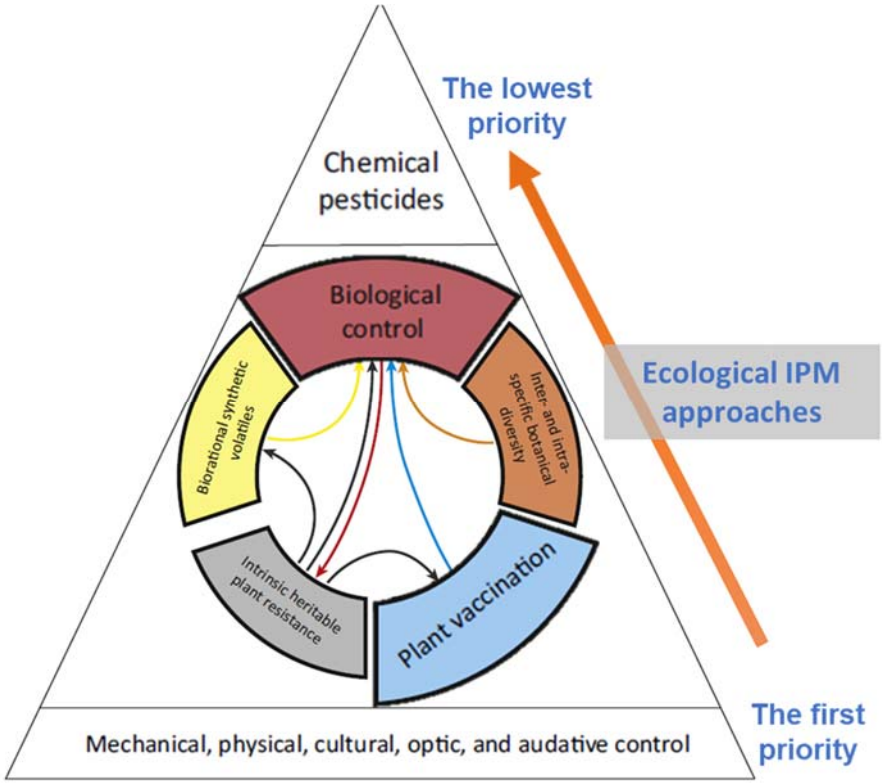

Figure 1 *IPM pyramid showing the most important pest management elements*
Diverse crop protection measures and interactions among ecological approach elements are illustrated. Modified from Stenberg (2017).

widely accepted. Still, the efficiency of IPM is yet being questioned and requires more research on the synergistic application of various actions and the relationship among the major elements. Stenberg (2017) illustrated the major elements of IPM into the 'IPM Pyramid,' showing the priorities and the interactional influences among the elements (Fig. 1). Among them, biological control, which is suppressing the plant damage and the population of pest, weed, and pathogen by utilizing living organisms (Eilenberg et al., 2001), is designated as the most sensitive element that is influenced by almost every other IPM element (Stenberg, 2017). Therefore, it will be essential to know how this element is affected by current plant protection measures.

For sustainable plant protections, we need to decipher a plethora of plant defense mechanisms employed by plants to deal with simultaneous attacks by various arthropods and pathogens. Resistance of plants to herbivores can be present independent of damage (constitutive resistance) or induced by herbivory (inducible resistance). According to their ecological impact, they can be direct resistance, which directly affects herbivores, and indirect resistance, which positively affects the performance of herbivore natural enemies (Schoonhoven et al., 2005). In the early 1970s, the role of herbivory-induced resistance of plants started to be reported (Green & Ryan, 1972). And now, it is widely recognized that the majority of plant secondary metabolites, enzymes, and proteins associated with plant defenses can be induced by biotic factors, especially by insect herbivory (Schoonhoven et al., 2005).

The induction of plant defense is regulated by small signal molecules called phytohormones such as jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA), and ethylene (ET). In general, the jasmonic acid (JA)-dependent signaling pathway is effective against necrotrophic pathogens and leaf-chewing insects, while Salicylic acid (SA)-dependent signaling is effective against biotrophic pathogens. These two key regulatory pathways often interact antagonistically. Other phytohormones also affect them, which build up complex but highly flexible plant defense networks and together determine the specific nature of plant response (Gruden et al., 2020; Pieterse et al., 2012).

Some key metabolites triggered by plant defense are volatile compounds. Plant volatile compounds are lipophilic compounds with a molecular weight under 300 with high vapor pressures, and they are stored and emitted in leaves, roots, and flowers (Dudareva et al., 2004). Volatiles emitted from vegetative organs are involved in not only constitutive defense but also inducible defenses. Green leaf volatiles (GLVs), Terpenes, and Phenylpropanoid volatiles are known to increase in quantity upon tissue damage. Their *de novo* biosynthesis, storage, and emission of plant volatiles are spatially and developmentally regulated by gene expression, in which loads of enzymes are involved. The herbivore-induced-plant-volatiles (HIPVs) are

complex blends of hundreds of compounds that change dynamically yet possess high specificity. The biosynthesis and emission of HIPVs are activated by different feeding, oviposition, and attack by insect pests and pathogens; therefore, this specificity offers a reliable cue for the natural enemies about the attacker. It ultimately acts as pest repellents or herbivore enemy attractants, minimizing the plant damage. In addition, predators, parasitoids or herbivore pathogens, other herbivores, pollinators, and neighboring plants respond to HIPVs. Therefore, knowledge of the mechanisms and the ecological function of plants' ability to facilitate volatiles is necessary for sustainable agriculture (Koeduka et al., 2020). Recent state-of-the-art genetic and chemical techniques enabled unraveling the related plant physiology and gene expression for signal perception and plant volatile emission. However, plants respond to stimulations from not only aboveground organisms but also diverse belowground organisms such as insects, nematodes, and microorganisms (Dicke & Lucas-Barbosa, 2020).

Beneficial microorganisms such as growth-promoting bacteria and fungi, arbuscular mycorrhizal fungi (AMF), and nitrogen-fixing rhizobia are known to positively affect the water and nutrient uptake or the resistance against stress and pathogens. However, our understanding of the effect of root-associated beneficial microbes on plants' indirect defense has only developed in the recent 20 years (Tao et al., 2017). Recent studies show that several soil microbes can trigger induced systematic resistance (ISR) against pathogens and herbivory insects at aboveground (Pieterse et al., 2016; Pineda et al., 2010) and belowground (Martínez-Medina et al., 2017b). ISR is to achieve high resistance of plants against subsequent attackers by being exposed to initial attacks such as necrotrophic pathogens (Kessler & T. Baldwin, 2004) or beneficial microbes (Pieterse et al., 2014). It establishes a 'primed' state categorized to the plant vaccination in the IPM pyramid (Fig. 1).

An increasing number of case studies reported the indirect plant defense influenced by mycorrhizae and *Trichoderma* spp. Mycorrhizae affected the herbivore enemies' preference behavior (Guerrieri et al., 2004; Hoffmann et al., 2011a; Schausberger et al., 2012), density (Gange et al., 2003; Schreck et al., 2013), and developmental and reproductive traits (Hempel et al., 2009; Hoffmann et al., 2011b; Moon et al., 2013). When inoculated with *Trichoderma* spp., the attraction of aphid parasitic wasp *Aphidius ervi* was significantly increased (Battaglia et al., 2013; Coppola et al., 2019). Moreover, alteration in foliar arthropod communities in a maize field attributing to *T.harzianum* was also reported, showing increased pest regulating arthropods (Contreras-Cornejo et al., 2020). However, according to meta-analytic studies on this topic, the effects of microbes on plant defense against chewing insects, phloem feeders, mesophyll feeders (Koricheva et al., 2009), and the response of natural enemies (Tao et al.,

2017) were inconsistent and markedly differed depending on the measured parameter and introduced fungal species, and arthropods species.

Moreover, recent studies on ISR have studied on a limited range of herbivores and natural enemies, mainly focusing on aphid-parasitoid wasps (Battaglia et al., 2013; Gadhave et al., 2016; Guerrieri et al., 2004; Hempel et al., 2009; Pineda et al., 2013) and spider mite-predatory mites (Hoffmann et al., 2011a, 2011b; Schausberger et al., 2012). Only a few studies reported the effect on leaf-mining insects (Gange et al., 2003) and other predators, *Macrolophus pygmaeus* (Battaglia et al., 2013), mutualistic predatory ants (Godschalx et al., 2015), and *Orius sauteri* (Ueda et al., 2013). Therefore, this limited amount of evidence cannot establish any generality of direction and the magnitude of the effects of soil microbes to the third trophic levels, leaving significant knowledge gaps (Tao et al., 2017). Therefore, these findings should be more elucidated by working on different biological systems (microbe, plant, herbivore, enemy), including a wide range of microbes and generalist natural enemies of herbivores.

This study aimed to assess the effect of two different root symbionts, AMF *Rhizophagus irregulari*s and *Trichoderma harzianum* strain T78, on the indirect defense of tomato plants challenged by generalist chewing insect *Spodoptera exigua*. Firstly, I trapped the VOCs emitted by tomato plant vegetative organs and analyzed them using Gas Chromatography coupled with Mass Spectrometry (GC-MS). Subsequently, microbial effects on biosynthesis and VOC regulation are investigated in transcriptional levels by targeting JA- and SA- pathway-associated genes. The performance of plants inoculated with the microbes and infested by *S. exigua* for 24 hours was compared with non-inoculated and non-herbivory treated plants. Lastly, I tried to elucidate the ecological impact of these changes by observing the behavioral response of the omnivorous predator, *Macrolophus pygmaeus*. I conducted Y-olfactometer bioassays, examining the attraction of *M. pygmaeus* toward the blends of HIPVs emitted by microbe-inoculated tomato plants upon *S. exigua* herbivory. I hypothesized that the colonization with beneficial fungi would modulate JA and SA signal transduction pathways and establish the primed status in tomato plants, which may enhance the indirect defense against a generalist herbivore. Therefore, the emission of significant HIPVs and associated gene expression levels were expected to increase in microbe-inoculated plants, significantly attracting the omnivorous predator to the HIPVs. Together, these results will reveal the interaction between soil symbionts and omnivorous insect predators, casting light on the potential application of beneficial microbes harmonized with biological control insects for more efficient IPM practices.

2. Species Description

2.1 Tomato (*Solanum lycopersicum*); importance and common pests

Cultivated tomato, *Solanum lycopersicum* L. cultivar Moneymaker, is one of the most commercially important vegetables consumed worldwide and has been used as a model species representing cultivated dicotyledonous plants (OECD, 2017). Tomato plants have various defense mechanisms against arthropod herbivores. Glandular trichomes and non-glandular trichomes on the foliage and stems form the first line of their defenses (Kennedy, 2003). Tomato's most important insect pests include several noctuid caterpillars, beetles, thrips, aphids, and whiteflies. *Spodoptera exigua* is one of the tomato's pests that causes significant yield losses (OECD, 2017). Jasmonic acid and salicylic acid signaling pathways are known to modulate the resistance of tomato plants to herbivores and pathogenic attacks. Sometimes the interaction between these pathways attenuates the resistance of tomato plants to *Spodoptera exigua* (Thaler et al., 2002). The defense-related gene expression and HIPV profiles after herbivory of various insect herbivores are sufficiently reported (van Poecke & Dicke, 2004; Zebelo et al., 2014). Therefore, the tomato plant-*S. exigu*a system is a suitable model for beneficial microbe-induced defense performance against chewing herbivores.

2.2 Arbuscular Mycorrhiza Fungus (*Rhizophagus irregularis*)

Arbuscular mycorrhizas are the most widespread symbiotic association between specialized soil fungi and plant roots. Mycorrhizal fungi are divided into two broad categories: Ectomycorrhizas (EMs) and endomycorrhiza, subdivided into orchid, ericoid, and arbuscular mycorrhiza fungi (AMF, also called Vesicular-arbuscular mycorrhizas). All AMF and some EMs are incapable of independent growth and require a host for their development (Brundrett, 2002). AMF involve fungi of the phylum Glomeromycota, order Glomales, and their fungal hyphae enable plants' effective acquirement of soil nutrients and water (Bitterlich et al., 2018). The hyphae reach the inner root cortex and develop a highly branched structure, called arbuscules, inside the cell lumen for nutrient and signal exchange (Bonfante & Genre, 2010). Plants offer photosynthates to fungi, and mycorrhizal fungi enhance the uptake of mineral resources and water absorption by plants, promoting plant growth and development.

In addition, AMF symbiosis is reported to alter the plant defense against pathogens and herbivores by triggering a mild immune response (Bonfante & Genre, 2015), which modulate the level of phytohormones and metabolites. Consequently, plants are rendered more effective

at responding stronger and faster to subsequent attackers in the roots and shoots (Kaling et al., 2018; Koricheva et al., 2009). Therefore, mycorrhizal fungi change the plant physiology with which they are directly associated and shape the multi-trophic interaction surrounding the plant (Ferlian et al., 2018).

2.3 Plant Growth Promoting Fungus (*Trichoderma harzianum*)

Opportunistic and avirulent plant root symbionts *Trichoderma* spp. (teleomorph *Hypocrea*, Family: Hypocreaceae) are found in most soils and rhizosphere or as endophytes. They kill other pathogenic fungi by emitting cell wall-degrading enzymes and compete with other phytopathogenic microbes for space and nutrients in the ground. They are also widely acknowledged to promote plant growth, crop productivity, and higher tolerance to abiotic stresses. Based on their versatile use and effectiveness in phytopathogenic fungi control, *Trichoderma* spp. have been commercially used as a potent biological control agent since the 1930s (Guzmán-Guzmán et al., 2019; Saba et al., 2012).

Recently it has been reported that *Trichoderma* spp. stimulate ISR in plants by altering metabolites and transcript levels, which affect various insect herbivores (Coppola et al., 2019; Jafarbeigi et al., 2020; Muvea et al., 2014) and plant-pathogenic microorganisms and viruses (Harman et al., 2004). These influences are anticipated to modify foliar arthropod communities (Contreras-Cornejo et al., 2020).

2.4 Beet Armyworm (*Spodoptera exigua*); origin, pest status, and control

The beet armyworm, *Spodoptera exigua* Hübner 1808 (Lepidoptera: Noctuidae), is native to Southeast Asia but increases the distribution to at least 101 countries (NVWA, 2017), migrating and overwintering in different regions of the world (CABI, 2017; Xia-lin et al., 2011). The *Spodoptera* species are polyphagous and have a broad host range, including economically important crop plants, causing economic losses. In the American continents, *S. exigua* is one of the most destructive pests in the field and greenhouse. In Europe, it is a significant greenhouse pest overwintering in greenhouses because the summer temperatures in most European countries do not support one complete life cycle outdoors (NVWA, 2017). However, *Spodoptera* spp. have evolved to have high resistance to insecticides (Caccia et al., 2014) and requires sustainable management methods such as biological controls. Studies on natural enemies of *S. exigua* on sugar beet (Darsouei et al., 2018; Ehler, 2004) and cotton (Ruberson et

al., 1994) listed the taxonomy of predators, parasitoids, and pathogens and their impact on the survival and reproduction of *S. exigua* found in the USA and northeastern Iran. These include various species in Heteroptera as predators of eggs and young larvae and parasitoids in Diptera and Hymenoptera. These species are non-native species in many other countries globally, and little information is known on natural enemies in different continents. Darsouei et al. (2018) pointed out that the majority of their reported natural enemies were generalists, and when the specific, imported biological control agents are missing, the control of the introduced pest, *S. exigua*, would be done by generalist enemies. Therefore, against the greenhouse pest species in many countries in Europe, unveiling associations with other greenhouse biological control agents will be necessary to achieve integrated pest management against these pests.

2.5 Mirid Bug (*Macrolophus pygmaeus*); description and common usage in Europe

Zoophytophagous, piercing-sucking mirid bug, *Macrolophus pygmaeus* (Rambur 1839) (Hemiptera: Miridae), feeds on plant tissue, pollen and nectar, and at the same time acts as a generalist predator of a wide range of agricultural pests such as whiteflies, aphids, thrips, mites and moth species. They have been found on plants from Solanaceae, Asteraceae, and Lamiaceae families (Martinez-Cascales et al., 2006; Sanchez et al., 2012). In Southern European countries, they occur naturally in the crop fields and greenhouses, and the success of their colonization depends on the vegetation and the use of insecticides. Since the early 1990s, *M. pygmaeus* have been sold and used as commercial biological control agents and are employed mostly in European greenhouses, especially standardized in Northwestern European tomato greenhouses (EPPO, 2021; Sanchez et al., 2012). Although the attraction of predatory mirid bugs toward HIPVs was tested in a few herbivore and plant species (De Backer et al., 2015b; Moayeri et al., 2007; Saad et al., 2014), their olfactory response to plant volatiles infested by caterpillars was not studied.

3. Material and Methods

3.1 Plant, Fungal, and Insect Material

Tomato plant (*Solanum lycopersicum*, cv. Moneymaker) was used in the greenhouse experiment and bioassays. The source of tomato seeds is Intratuin B.V (Woerden, The Netherlands) and all seeds used were from an identical package. For stratification, I sterilized the surface of the seeds by putting 15 seeds in each 50 mL Falcon tube with 40 ml of 10% (v/v) sodium hypochlorite (NaOCL, 12% ChemSolute, Th. Geyer, Berlin, Germany) and shaking for three minutes. Subsequently, I drained and rinsed the seeds with warm tap water and repeated shaking and rinsing the seeds with 40 ml of warm water four more times. The seed germination took place in a plastic box filled with fine-grained vermiculite moistened with tap water and closed with a perforated clear plastic lid. The seed germination boxes were covered with aluminum foil to block the light and placed in an incubator at 28 °C. After three days, the aluminum foil covers were removed. The boxes were placed in a plant growth chamber (E-36L, Percival Scientific, Perry, United States) at 24 °C during the daytime and 21 °C at night with a 14-hr photoperiod and 60% RH conditions. After four nights, the seedlings were used for the experiments.

The inoculum of *Rhizophagus irregularis* (DAOM197198-research grade, 1 million spores in 100 g attapulgite powder, batch S.380 – 02.2021) and heat-sterilized carrier material were purchased from SYMPLANTA GmbH & Co. KG (Darmstadt, Germany) and stored at 8 °C until used. The inoculum of *Trichoderma harzianum* isolate T-78 (CECT 20714, Spanish Type Culture Collection) was prepared from a strain that was maintained on 5% w/v potato dextrose agar (PDA, Sifin diagnostics, Berlin, Germany) plates. I autoclaved a mixture of 7 g of vermiculite and 19 g of oat flake with 20 ml of water in an Erlenmeyer flask and placed one mycelium plug of 1.5 × 1.5 cm inside the flask, and closed it with a cotton lid. One flask of inoculum and one flask with autoclaved carrier material without fungi were incubated at 28 °C in the dark for six days.

As generalist chewing herbivores, *S.exigua* caterpillars were reared and prepared. The eggs of *S.exigua* were purchased from Entocare Biologische Gewasbescherming (Wageningen, The Netherlands) and reared on an artificial diet (Hoffman et al., 1966). Several generations of *S.exigua* were maintained in a growth chamber (E-36L, Percival Scientific, Perry, United States) with a 12-hr photoperiod at 25 °C, 45% RH conditions.

Three hundred adult *M. pygmaeus* were purchased from Katz Biotech AG (Baruth, Germany) in April 2021 and reared until the next generation grows to adult insects. For approximately

eight months, the adult and nymph insects were reared in insect net cages on pregrown tomato plants. The omnivorous insects were offered with *Sitotroga* eggs (Katz Biotech AG, Baruth, Germany), 1:4 diluted honey water (v/v) soaked on a piece of cotton pad, and organic bee pollen (Biojoy GmbH, Nürnberg, Germany) as Vandekerkhove and De Clercq (2010). The insect cages were placed in a plant growth chamber (E-36L, Percival Scientific, Perry, United States) at 24 °C during the daytime and 21 °C at night with a 14-hr photoperiod and 60% RH condition. Tomato plants in the cage were watered every two days and fertilized by Hoagland nutrient solution (Hoagland & Arnon, 1950) once per week. The other food material of *M.pygmaeus* were replaced every two days.

3.2 Fungal Inoculation and Plant Growth Condition

Before the prepared seedlings were transplanted into 1-L pots, the soil (Floradur® B Pot Clay Medium, Floragard Vertriebs-GmbH, Oldenburg, Germany) was mixed with washed sand (1:1 v: v) and autoclaved. I aimed to have 30 AMF spores per milliliter of soil mixture and put the weighed *R.irregularis* inoculum in the planting hole to provide higher initial concentrations than homogenizing the soil with the inoculum (http://www.symplanta.com/faqs). Therefore, the soil mixture for *R.irregularis* treatment was firstly mixed with carrier material of *T.harzianum* inoculum (1g inoculum / 1 kg of soil mixture), and 3 g of *R.irregularis* inoculum was put inside the planting hole of 1L soil. For the *T.harzianum* treatment, I mixed the prepared inoculum with the soil to reach a final density of 1 × 106 conidia g−1 '97' and put 1.5 g of carrier material of *R.irregularis* inside the planting hole. The soil for control treatment was prepared in the same way with both carrier materials. Subsequently, I transplanted the tomato seedlings into the planting whole and covered the topsoil with sterilized sand to a height of approximately 0.5 cm.

The seedling pots were then placed in a greenhouse (3.8 m × 6 m) with supplemental LED lighting of 3500 k and 80 CRI (RUBOL JOSEPHINE 135W V2 LUMINUS CXM-32 DIY KIT, Rubol, Dronten, The Netherlands) at a 16 h: 8 h day: night cycle and ventilation system in 10 min on and 10 min off. The greenhouse air temperature and relative humidity were recorded for five weeks from April to May in 2021. During daytime was 25.82±3.79 °C, 46.87±4.76 %RH and in the dark was 22.29±2.84 °C, 49.29±4.19 %RH. The pots were bottom watered via separate plant saucers every second day with 50-ml tap water and once every six days with Hoagland nutrient solution (Hoagland & Arnon, 1950). Every week the position of pots was rotated to avoid spatial effect inside the greenhouse.

3.3 Shoot Herbivore Infestation and Sampling

To compare the herbivore-induced plant defense with the constitutive defense of tomato plants, half of the tomato plants in each microbe-treatments and control groups were infested by three third-instar *S.exigua* larvae for 24 hr. One day before the herbivory took place, the second- and third-instar larvae of *S.exigua* were moved from the artificial diet and placed in plastic containers with prepared tomato plant leaves for acclimatization. The containers with caterpillars were placed in a greenhouse next to the experimental chamber overnight. To confine the herbivores on a tomato leaf, I used clip cages made of foam-floating tubes and microperforated plastic flower sleeves fixed on a wooden stick (Fig. 2 a). Three third-instar larvae were placed on the terminal leaflet and two primary leaflets inside each clip cage from 9 am for 24 hr. Non-herbivory-treated tomato leaves were closed with clip cages for 24 hr as well.

After removing the herbivores from the clip cages, plant volatile compounds were trapped by placing polydimethylsiloxane (PDMS) tubes in the clip cages (Fig. 2 b) from 10 am for four hours. The temperature was 25.61 ±1.19 °C, and the humidity was 42.27±2.09%RH while the volatiles were trapped. Subsequently, tomato leaves were harvested using a disposable scalpel, wrapped with aluminum foil, and immediately froze with liquid nitrogen, according to Bandoly and Steppuhn (2016). The samples were stored at -80 °C until further analyses. To evaluate final fungal colonization levels, soil and root samples were harvested and stored in 50 mL Falcon tubes at 4 °C.

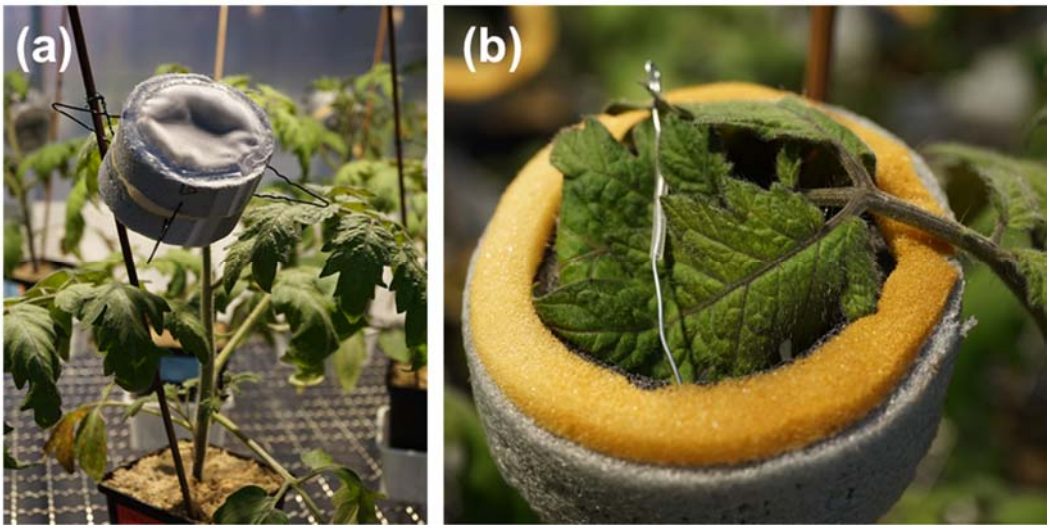

Figure 2 *Clip Cages Used in this Experiment*

For confining *S.exigua* and colleting leaf volatiles (a) two clip cages are fixed on a wooden stick closing three tomato leaflets inside and (b) PDMS tubing cuts were placed inside the clip cages for leaf volatile trapping.

3.4 Passive Volatile Trapping, Analysis using GC-MS, and Data Processing

To study the effect of symbiotic fungi on the volatile emission of tomato plants after 24-hour herbivory of *S. exigua*, leaf volatiles were passively sampled using PDMS cuttings following a protocol described by Kallenbach et al. (2015). PDMS tubing is widely used for volatile collection due to its cost-effective and versatile use and the possibility of retaining the VOCs at freezing temperatures until thermal resorption (Tholl et al., 2021). Silicon tubing (ST; Rotilabo®, inner-Ø 1 mm, outer-Ø 1.8 mm, Car Roth GmbH+Co.KG, Karlsruhe, Germany) was cut into 5 mm and soaked in 4:1 acetonitrile: methanol(v/v) for three hours at room temperature. After decanting the solvent, the STs were heated in a heating oven under nitrogen flow (5 L/min) for 1.5 hr at 210 °C. Cooled down STs were transferred into brown 4-ml glass vials, sealed with PTFE tape, and stored at -20 °C until used. I placed two PDMS tubes as technical replicates on wire cuttings inside the insect clip cages with tomato leaves (Fig. 2 b) and let the PDMS absorb VOCs for 4 hours. The background volatiles (BLANK) were trapped in the same way by placing PDMS tubes at each corner of the greenhouse without adjacent plants. Each set of two PDMS tubes were put in individual brown glass vials with a screw cap and immediately placed in an ice box and later stored at -20 °C until analyzed. I transferred each PDMS tube to empty stainless-steel tubes (MARKES), and the PDMS samples were analyzed using a thermal desorption-gas chromatograph-mass spectrometer (TD-GC-MS). TD-GC-MS consisted of a thermo desorption unit (MARKES, Unity 2, Llantrisant, UK), an autosampler (MARKES, Ultra 50/50), and a gas chromatograph (Bruker, GC-456, Bremen, Germany) connected to a triple-quad mass spectrometer (Bruker, SCION). A DB-WAX column (30 m x 0.25 mm inner diameter x 0.25um film thickness, Restek) was employed, and helium was used as a carrier gas at a constant flow rate of 1 ml/mi. VOC desorption and MS conditions are written in Supplementary Material 1. The features of peaks were detected by using XCMS online website (https://xcmsonline.scripps.edu/) with parameters in Supplementary Material 1 Table 3.

High peak intensities analyzed by GC-MS appeared in BLANK samples; thus, I subtracted the average peak intensities of BLANK samples from the measured values of each detected feature. The values lower than average BLANK peak intensities were replaced by NA and excluded for statistical analyses. Features that had NA in more than 50% of volatile samples were excluded, and features annotated by the NIST (National Institute of Standards and Technology) library as Cyclooctasiloxane and 1,3,5-Trioxepane were not included to exclude volatiles from silicon

and plastic material. Remained 64 features were tentatively predicted by using NIST Library Search on the Brucker workstation if possible.

3.5 Extraction of Tomato Leaf total RNA and cDNA Synthesis

To extract the total RNA from frozen tomato leaves, leaf materials were manually ground and homogenized on liquid nitrogen using stainless-steel mortars with a PE insulating jacket and porcelain pestles. Approximately 100 mg (fresh weight) of ground leaf material was transferred into 2.0 mL Eppendorf tubes and stored at -80 °C for further extraction. Total RNA extraction and quality check RNA were performed according to Oñate-Sánchez and Vicente-Carbajosa (2008) with slight modifications (Supplementary material 2). The RNA extraction and cleaning results were checked by gel-electrophoresis (1% agarose gel, 1× TAE buffer, 5× Loading Dye QIAGEN), and the concentrations were measured using Nanophotometer (P300, IMPLEN GmbH, München, Germany). First-strand cDNA was synthesized from 2 µg DNase free RNA according to a laboratory protocol (Supplementary material 2) using RevertAid H Minus Reverse Transcriptase (Thermo Scientific ™) and Prime Thermal Cycler (5PRIME/02, Techne®).

3.6 Differential Gene Expression Analysis Using RT-qPCR

To compare the expression level of indirect defense-related genes of tomato plants in each treatment, six gene-specific primer sets and a housekeeping gene (Tab. 1) were selected. As JA-pathway-related genes, two lipoxygenases (LOX) genes, one allene oxide synthase(AOS) gene, and one JA-induced terpene synthase (TPS) gene were used. LOX, LOXA, AOS2 which are components of octadecanoid signal transduction, are involved in the biosynthesis of JA and GLVs, and TPS5 engages in the biosynthesis of monoterpene Linalool (Cao et al., 2014). For SA- pathway-related genes, phenylalanine ammonia-lyase (PAL) and salicylic acid carboxyl methyltransferase (SAMT) were selected. PAL in phenylpropanoid pathways is involved in SA biosynthesis and SAMT modifying SA into MeSA, which becomes volatiles (Ament et al., 2004). I used 1µL cDNA as a template in a total amount of 10 µL using SsoAdvanced SYBR® Green Supermix(Bio-RAD) and selected primers. Bio-Rad Hard-Shell® 384 microplates (U.S.) and Bio-RAD Microseal® 'B' PCR Plate Sealing Films were used for preparation. In total, six to nine biological replicates of tomato RNA samples were analyzed with three technical replicates for each gene.

Reverse-transcription quantitative real-time PCR (RT-qPCR) was performed using C1000 Touch Thermal Cycler with CFX384™ Optical Reaction module for Real-Time PCR Systems (Bio-RAD) with following cycling program: 2 min 50 °C, 10 min 95 °C, 40 cycles of 15 sec 95 °C and 1 min 60 °C, followed by a melting curve analysis. The relative gene expression level (2-ΔΔct) was calculated on Microsoft Excel according to Pfaffl (2001), and expression levels of six targeted genes were normalized to SlEF mRNA levels.

Table 1 *Selected Genes for RT-qPCR and Sequence of the Primers*

Abbreviation	Target Gene	Sequence ID	Sequence (5'->3')
SlEF	tomato elongation factor 1α	X14449.1	Forward GATTGGTGGTATTGGAACTGTC Reverse AGCTTCGTGGTGCATCTC
PAL	*Solanum lycopersicum* phenylalanine ammonia-lyase 5	NM_001320040.1	Forward CGTTATGCTCTCCGAACATC Reverse GAAGTTGCCACCATGTAAGG
SAMT	*Solanum lycopersicum* S-adenosyl-L-methionine salicylic acid carboxyl methyltransferase	unknown	Forward GGGTTGTTCTTCTGGAGCGA Reverse CGCGTTAAAATCATTTCCAGGGA
LOX	*Solanum lycopersicum* lipoxygenase (LOX1.1)	NM_001247927.2	Forward GGTTACCTCCCAAATCGTCC Reverse TGTTTGTAACTGCGCTGTG
LOXA	*Solanum lycopersicum* lipoxygenase (LOX1.1)	NM_001247927.2	Forward GGTTACCTCCCAAATCGTCC Reverse TGTTTGTAACTGCGCTGTG
AOS2	*Solanum lycopersicum* allene oxide synthase 2 (AOS2)	NM_001287778.2	Forward AGATTTTCTTCCCGAATATGCTGAA Reverse ATACTACTGATTTCATCAACGGCAT
TPS5	Monoterpene (Linalool) synthesis	AAX69063	Forward CTTCGGATGAACTGAAAAGAGG Reverse GTGGAGAATTTTTGCTTTGAGC

3.7 Y-tube Olfactometer Bioassays

Bioassays were conducted to assess the olfactory response and preference of *M. pygmaeus* toward the volatile compounds of tomato plants under different treatments. Tomato seedlings and inoculums for the bioassays were prepared the same way as the greenhouse experiment but

transplanted in 100-ml pots to fit inside the odor source chamber. I put the combination of inoculums (*R. irregularis*, 0.3g; *T. harzianum*, 1g) and carrier materials (*R. irregularis*, 0.15g; *T. harzianum*, 1g) in the planting holes as following: Non-inoculated plants, both carrier materials; *R. irregularis*-inoculated plants, carrier material of *T. harzianum* and the inoculum of *R. irregularis*; *T. harzianum*-inoculated plants, carrier material of *R. irregularis* and the inoculum of *T. harzianum*. The plants were grown in the plant growth chamber (E-36L, Percival Scientific, Perry, United States) at 24 °C during the daytime and 21 °C at night with a 14-hour-photoperiod and 60% RH condition. Two plants of 27 to 36-day-old were paired by the most similar age and shoot biomass, then three third-instar larvae of *S. exigua* were let on the whole plants for 24 hr before the bioassay (Fig. 3 a). Adult female *M. pygmaeus* were isolated from the colony at least four hours before the bioassay (Fig. 3 b) and put individually in a plastic container with a perforated lid in a darkroom for stabilization. I used a Y-shaped glass tube (internal Ø 18 mm) consisting of one 140-mm-long entry arm and two 100-mm-long side arms at 110° angle apart. The air was generated by an air compressor (OLF2502, Jenpneumatic) and delivered through polyurethane tubes at an air flow of 500±50 ml/min adjusted with air flow valves. As an odor source, one entire potted tomato plant was placed inside each of two 1.5 L glass chambers with taps. In order to avoid the direct effect of soil microbes, the pot and soil were covered with aluminum foil. Airflow was measured with flow sensors (PFMV510-1, SMC, Japan, designed by J.Wilde and D. Veit) between each odor chamber and side arm of the Y tube. According to Takabayashi and Dicke (1992), the insect clade of *M.pygaeus* performed better in a vertical setup; thus, the Y-tube was fixed vertically using a clamp and a stand (Fig. 3 c). To exclude visual cues and spatial effect, the laboratory was kept as a darkroom. Additional lighting (LEICA KL1600 LED, the 4th level) illuminated the end of both side arms of the Y-tube, and the odor chambers were screened off.

A single female *M.pygmaeus* was introduced at the entry arm of the Y-tube and let crawl up and make the decision between two odor sources within 10 min. When the bug walked at least 4 cm up on one of the side arms, the decision was recorded. If it did not make a choice within 10 min, it was recorded as "No choice" and not used in the data analysis. The following odor source pairs were tested: Test 1, tomato plant versus empty glass chamber; Test 2, undamaged and non-inoculated tomato plant versus non-inoculated tomato plant after 24-hour herbivory of *S. exigua*; Test 3, non-inoculated plant after 24-hour herbivory versus *R. irregularis*-inoculated plant after 24-hour herbivory; Test 4, non-inoculated plant after 24-hour herbivory versus *T. harzianum*-inoculated plant after 24-hour herbivory; Test 5, *R. irregularis*-inoculated plant after herbivory versus *T. harzianum*-inoculated plant after herbivory. For each comparison, a

maximum of 10 observations was conducted at each time, and each test was replicated five to six times with new odor sources on different experimental days. Each insect and plant were used only once. After testing five insect individuals, the position of two odor source chambers was switched between left and right-side arms, and after every ten observations, the Y-tube and odor source chambers were cleaned thoroughly with Ethanol 70% and completely dried in a

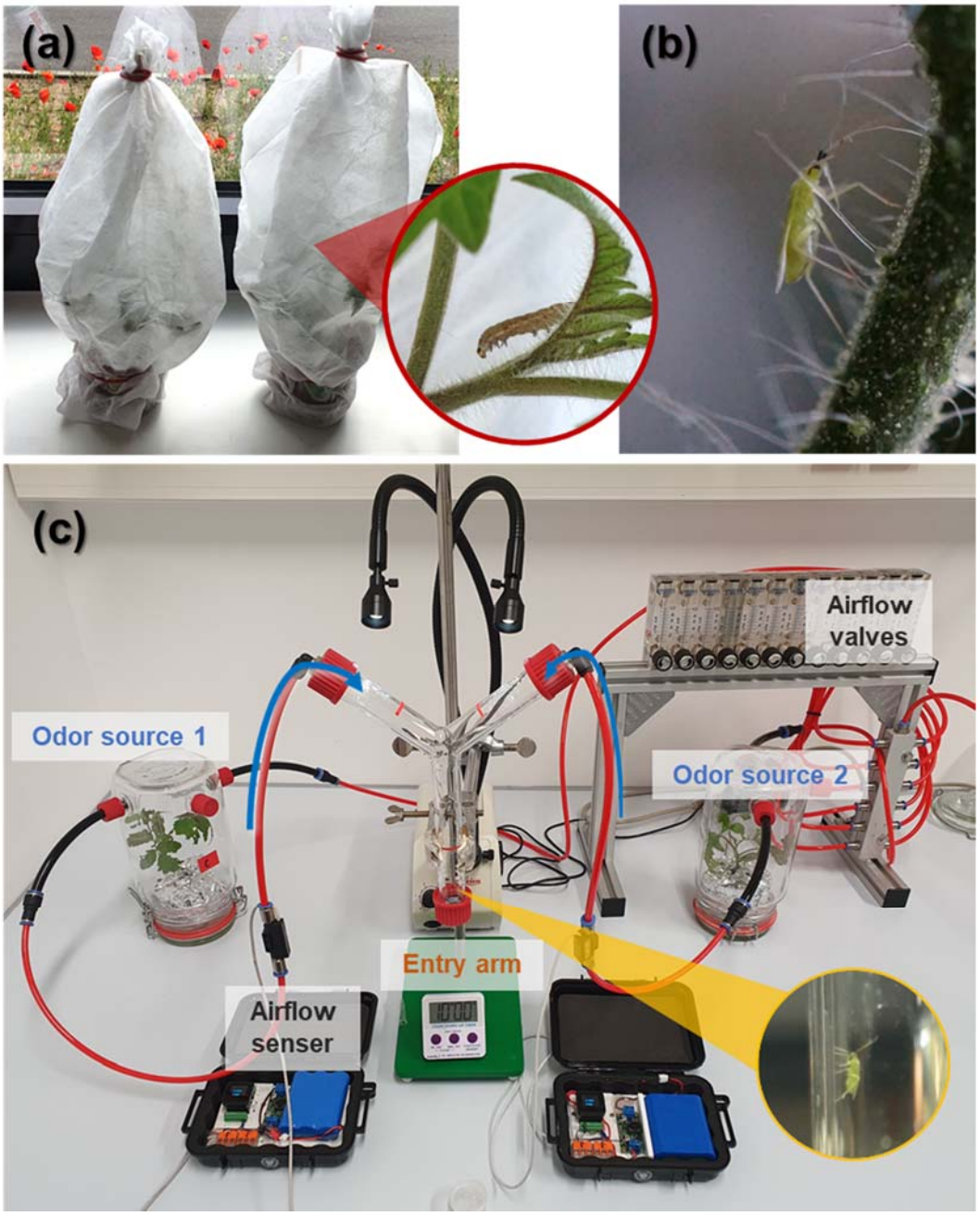

Figure 3 *Y-tube Olfactometer Assay Setting*

(a) *S.exigua* feeding on tomato plants for 24 hr, (b) an adult female *M. pygmaeus* distinguished by its inflated abdomen and oviposition, (c) a vertically fixed Y-shaped glass tube connected to two odor sources in glass chambers , and single *M. pygmaeus* was introduced to the entry arm.

drying oven. The bioassays took place between 10:30 am and 6:00 pm in a laboratory with a ventilation system and a cooling system, keeping the room at 24 °C. The five tests were conducted in random order to avoid any temporal effect, and 37 to 47 choices after excluding 'No choice' were recorded from each test.

3.8 Quantification of Colonization Rate

To evaluate the successful colonization of two fungal species in the rhizosphere of tomato plants, soil and washed roots of plants in each treatment were sampled after the greenhouse experiment and bioassays.

For *R. irregularis* evaluation, one of the most common microscopic-based observation methods was used. The roots were washed with deionized water and put in 15 mL Falcon tubes. The roots were incubated for 60 min with pre-heated 10% KOH (w/v, ROTH, Karlsruhe, Germany) in a water bath of 80 °C. After rinsing the roots with deionized water, I stained the roots with trypan blue (ROTH) in 50% lactic acid water (0.05% w/v) for 10 min at 80 °C. Approximately 30 root segments were cut from each root sample. The intensity of fungal structures of each 1-cm-long segment was evaluated using a five-class system according to Trouvelot et al. (1986) under a binocular stereo microscope (Leica DM 4000 B LED). The intensity of mycorrhizal colonization in the root system (M%) was used as the main parameter of root colonization rate, following the method of Mycocalc (https://www2.dijon.inra.fr/mychin tec/Mycocalc-prg/download.html). The root samples of non-*R. irregularis*-inoculated plants were also observed to confirm that there were no AMF structures. The efficient colonization of *R. irregularis* in the corresponding treatments was confirmed with an average intensity of the mycorrhizal colonization in the root system (M%) of 14.45% by observing 21 root samples out of 33 plants used for the greenhouse experiment and bioassays. By observing 14 root samples from Non-inoculated and *T. harzianum*-inoculated plants, the intensity of 2.28% on average was calculated.

For the quantification of *T. harzianum* in the rhizosphere soil samples, colony-forming units (CFUs) of *T. harzianum* grown on streptomycin containing PDA colored with rose Bengal (Applichem, Darmstadt, Germany) were counted. PDA 39 g and 50 mg of Rosa Bengal (Carl ROTH) were mixed with distilled water up to 1 L and sterilized. After cooling down the PDA medium, filter-sterilized (0.33 μm, LABSOLUTE) streptomycin sulfate 100 mg were mixed thoroughly and poured in Petri dishes under a laminar flow clean bench (SAFE 2020 Thermo Sientific™). I mixed 1g of dried soil samples with 9ml of sterilized water and did a five-times-

serial-dilution to achieve a one-million-fold diluted. The final 100 µl of soil diluted solution was dropped on the prepared PDA plates, and the plates were incubated at 28 °C in the dark for five days. After five days, CFUs grown on the plates were counted. The successful inoculation of *T. harzianum* in the *T. harzianum*-inoculated plants was confirmed with an average of 7.74 CFUs from 27 soil samples out of 34 plants used. Non-inoculate and *R. irregularis*-inoculated plants had on average 0.71 CFUs. Cross-contamination levels of both fungal species in undesired root and soil samples were regarded neglectable.

3.9 Statistical Analysis

Differences in measured peak intensities of detected volatile features and relative gene expression levels in each microbial and herbivory treatment were analyzed in R (R Core Team, 2021). A two-way analysis of variance (Two-way ANOVA) was used to determine whether there were statistically significant differences among microbial and herbivory treatments. In addition, in order to evaluate the microbial effect on the *S. exigua*-induced-defense, data from herbivory-treated plants were subgrouped and compared using One-way ANOVA. Levene's test was used to test homogeneity of variance across groups, and the normality test was conducted using the Shapiro test. To meet the assumptions, all data used were log-transformed, and when the prerequisites were not met, a Non-parametric Pairwise Wilcoxon test was used. Tukey multiple comparisons of means followed ANOVA to identify significant differences among treatments. Principal Component Analysis (PCA) was conducted in R to explain the variation in the volatile peak intensity data. Hierarchical clustering analysis illustrated as a heatmap was performed in MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/). Euclidean was used as a distance measure, and the clustering algorithm was ward. D. The selection made by *M. pygmaeus* in each test of bioassays was analyzed using the One-Sample Chi-squared test in Microsoft Excel. It was examined whether there were statistically significant differences between selection percentages of two odor sources in each test.

4. Results

This study analyzed the effect of soil microbial symbiosis on the tomato plant indirect defense using the following three approaches in each chapter: 4.1, volatolomics; 4.2, transcript levels; 4.3, the behavioral response of *M. pygmaeus*. In each analysis, I focused on how *S. exigua* caterpillar feeding for 24 hours changes the volatiles, genes expression levels, and behavioral response of the generalist predator *M. pygmaeus* and subsequently, how the microbial association with *R. irregularis* or *T. harzianum* contributes to the changes.

There were six different tomato plant treatments: a control treatment without fungal association and not damaged by *S. exigua* feeding (**Non-inoculated**), tomato plants with no fungal association and infested by *S. exigua* for 24 hours (**Non-inoculated + *S. exigua***), tomato plants inoculated with *R. irregularis* and not infested by *S. exigua* (***R. irregularis***), tomato plants inoculated with *R. irregularis* with *S. exigua* herbivory of 24 hours (***R. irregularis* + *S. exigua***), tomato plants inoculated with *T. harzianum* without *S. exigua* herbivory (***T. harzianum***), tomato plants inoculated with *T. harzianum* and fed upon by *S. exigua* for 24 hours (***T. harzianum* + *S. exigua***).

4.1 Tomato Leaf Volatiles

I aimed to investigate whether *S. exigua* herbivory and microbial association with *R. irregularis* or *T. harzianum* in the soil would affect the plant leaf volatiles. Tomato leaf volatiles were trapped using PDMS tubes for 4 hr from non-herbivory treatments and herbivory treatments after 24 hr of *S. exigua* herbivory, respectively. BLANK samples were collected to consider background noises. After excluding features with high background noises (see text 3.4), 64 features remained and were used for further analyses, including the following indirect-defense-related volatile compounds (see Tab. 2): Monoterpene (F21, F22, F23, F24), Sesquiterpene (F17, F18, F25, F26, F27), Methyl salicylate (MeSA; F28) and GLVs (F9, F10, F12). Because the rest of the features were not matched to mass spectra of known compounds, they remained elusive but are included for statistical analyses.

Two-way ANOVA showed a significant effect of herbivory, microbe, and the interaction effect of these factors in some of the compounds (Tab.1). The significant interaction effect ($p_{H\times M}$) was found only in F2, F3, and F8, while marginally significant ($p<0.1$) interaction was observed in F1, F7, F17, and F18. The effect of *S. exigua* on the volatile emission is demonstrated with Two-way ANOVA and principal component analysis (PCA) (see text 4.1.1), and the effect of the microbial colonization is analyzed by ANOVA and clustering analysis (see text 4.1.2).

Table 2 *The List of 64 Volatile Features used and ANOVA Results*

The feature number corresponds to the features shown in Figures 4 and 5. Their corresponding mass to charge ratio (m/z) and retention time (rt) in minutes are offered and predicted volatile compound names according to the NIST library are given when possible. Two-way ANOVA with Herbivory (H) and Microbe (M) as factors was performed on the average peak intensity, and the F ratio is given with the following p values: · (p<0.1), * (p<0.05), ** (p<0.01) and *** (p<0.001). Additionally, to test the microbial effect on the *S. exigua*-induced volatile emission, the average peak intensity of volatiles from *S. exigua* infested plants were compared by One-way ANOVA. When a non-parametric test was required and performed by Pairwise Wilcoxon Rank Sum Test, it is indicated as W. ANOVA = analysis of variance

Feature			Two-way ANOVA			ANOVA upon herbivory	Annotation
	m/z	rt	F_H	F_M	$F_{H\times M}$	F	
F1	43.21	2.06	0.12	1.05	**3.35·**	**3.38·**	
F2	74.06	2.08	0.14	0.83	**5.40***	1.69	
F3	88.09	2.18	**6.31***	**2.08**	**5.29***	1.5	
F4	223.07	2.18		W		**4.27***	
F5	71.09	2.74		W		0.18	Butanoic acid, propyl ester
F6	297.16	2.93	<0.01	0.89	2.43	**3.09·**	
F7	371.11	3.29	0.22	1.5	**2.92·**	3.72	
F8	116.04	3.57	0.3	2.3	**4.29***	**4.15·**	
F9	67.12	3.76	**49.02*****	0.71	0.98	0.61	unknown GLVs
F10	67.11	4.09	**57.57*****	1.05	1.59	0.52	unknown GLVs
F11	299.23	4.46		W		W	
F12	67.12	4.5	**76.96*****	2.36	0.26	0.53	unknown GLVs
F13	140.98	4.56		W		**2.95·**	
F14	101.01	4.76		W		**3.17·**	
F15	73.08	4.91		W		**3.4·**	
F16	158.96	4.96		W		**4.98***	
F17	102.08	5.25	0.18	2.24	**2.69·**	**8.64****	unknown Sesquiterpene
F18	91.09	5.4	1.28	2.12	**2.65·**	2.33	unknown Sesquiterpene
F19	94.04	8.34		W*		**6.65***	
F20	74.12	9.91		W*		**4.37***	
F21	74.07	3.08	1.28	1.07	0.31	0.21	unknown Monoterpene
F22	93.08	3.23	0.86	0.13	0.59	0.27	beta.-phellandrene
F23	111.99	3.27		W		0.43	unknown Monoterpene
F24	209.01	3.91		W		2.67	unknown Monoterpene
F25	147.11	5.39	0.239	0.01	1.96	1.36	Caryophyllene
F26	93.11	5.87		W		W	Humulene
F27	159.11	6.1		W		0.47	unknown Sesquiterpene
F28	120.06	6.63	0.63	0.62	0.68	0.87	Methyl Salicylate
F29	110.96	2.05		W		0.68	
F30	71.09	2.06		W		0.38	
F31	59.08	2.12		W		0.67	
F32	74.07	2.55	0.05	0.24	0.84	0.08	
F33	297.09	2.61	0.04	0.89	2.41	2.74	
F34	253.14	2.65	0.37	0.58	0.68	1.45	
F35	144.97	2.65	0.84	1.53	2.29	1.95	
F36	91.03	3.37	0.12	0.43	1.46	0.67	
F37	371.11	3.43	2	0.19	0.82	1.73	
F38	415.11	3.52	0.11	1.21	0.17	1.53	
F39	119.08	3.56		W		0.13	
F40	135.02	3.95	0.17	0.24	1.16	0.67	
F41	74.07	4.13	0.02	1.71	0.2	0.66	
F42	402.09	4.16	0.14	1.64	0.22	0.59	
F43	75.07	4.18		W		W	

Table 2 (continued)

Feature			Two-way ANOVA			ANOVA upon herbivory	Annotation
	m/z	rt	F_H	F_M	$F_{H \times M}$	F	
F44	209.05	4.23	0.02	0.07	1.35	1.09	
F45	207.09	4.26	0.17	0.43	2.31	1.2	
F46	193.02	4.61		W		1.67	
F47	267.05	4.65	0.04	1.1	0.66	0.78	
F48	341.07	4.9	1.53	1.14	0.01	0.33	
F49	5.02	5.02		W		W	
F50	5.37	5.37	0.01	2.39	0.1	1.18	
F51	46.23	5.44	0.62	0.56	0.86	<0.01	
F52	105.03	5.53		W		W	
F53	5.79	5.79		W		W	
F54	128.03	6.41	0.1	1.62	0.08	1.08	
F55	72.13	6.52		W		0.29	
F56	145.07	8.41	0.19	0.87	1.37	2.07	
F57	224.05	9.11		W		W	
F58	129.98	9.78	0.18	0.57	1.59	1.17	
F59	175.05	9.84	3.53	0.91	0.95	1.8	
F60	197.22	10.12	0.32	0.01	1.01	0.38	
F61	274.36	10.24		W		0.79	
F62	198.09	10.39		W		W	
F63	76.12	10.49	0.02	0.17	0.62	0.74	
F64	185.16	10.58		W		0.05	

4.1.1 Effect of *S. exigua* Herbivory on Plant Volatiles

Volatile samples of *S. exigua*-herbivory-treatments were distinguished from non-herbivory-treatments when compared with a principal component analysis (PCA) (Fig. 4). The first and second principal components together explain 33.36% variance, and the score plot shows a clear separation of herbivory treatments (▲ in Fig. 4) from treatments without herbivory (● in Fig. 4). Due to the high variance in Herbivory treatment samples, it was not clear which principal component attributes to the effect of each treatment.

F5, F9, F10, and F12 had positive loadings on both PC1 and 2, pointing to the group of *S. exigua* herbivory treatments. These unknown GLV compounds F9, F10, and F12, showed significant herbivory effects in Two-way ANOVA, and Tukey posthoc tests (data not shown but see Fig. 5) revealed significantly higher emission of these compounds in *S. exigua* herbivory treatments. This result corresponds to the clustering of most herbivory-treated samples and the correlation with the cluster of these four compounds (Fig. 5: A_1 and A_2). The group of F5, F9, F10, and F12A were negatively or not correlated with other loadings pointing to non-herbivory

treatments on the negative side of PC1. However, these features had no significant herbivory effect in either Two-way ANOVA or Pairwise Wilcox tests (Tab. 2). Unknown sesquiterpene F17 and F18 were not correlated with herbivory-treatments, and the peak intensities of detected monoterpenes and most of the sesquiterpenes were not significantly different between herbivory and non-herbivory treatments (Tab. 2).

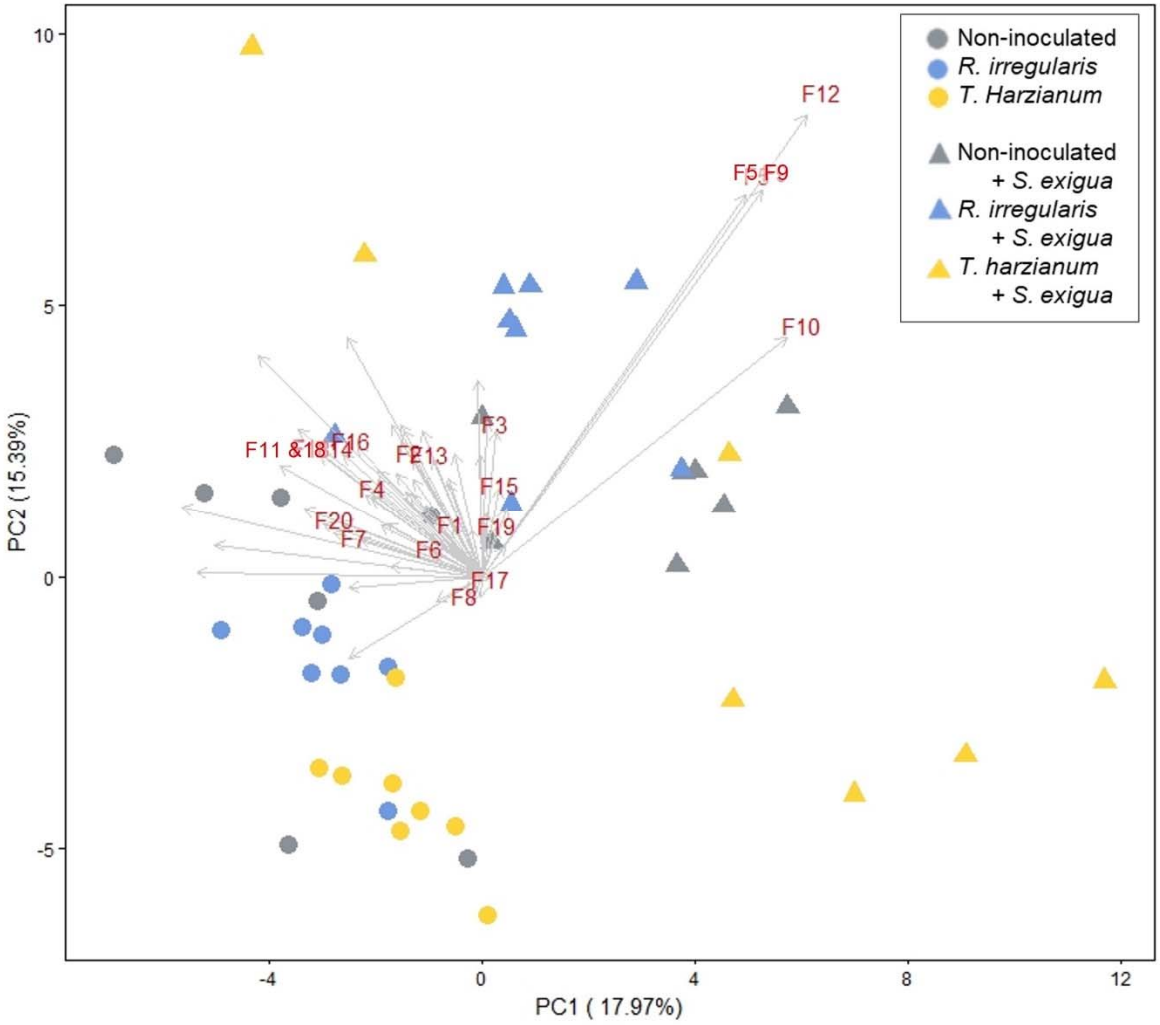

Figure 4 *Principal Component Analysis of tomato plant volatiles*

PCA shows firm grouping made by *S. exigua* herbivory: ●, volatiles from tomato plants without herbivory; ▲, volatiles from tomato plants upon *S. exigua* herbivory for 24 hr. Symbols represent biological replicates of each treatment with colors showing microbial treatments. Arrows represent the loadings of each volatile features and the loadings labeled by their feature names are significant features in Table 1. The corresponding loading shows the contribution of each feature to PC1, PC2 and clustering of each treatment. Loadings shown are multiplied by 20 times for visualization. Proportion of variance explained by PC1 and PC2 are written as a percentage on each axis. Peak intensity values used were blank-subtracted and log-transformed.

4.1.2 Effect of Microbes on the Constitutive and Herbivore-Induced Volatiles

I investigated whether the microbial association in the root area would affect the shoot volatile profiles. There was no significant microbial effect in Two-way ANOVA (Tab. 2) and no distinct separation among microbial treatments in a principal component analysis (Fig. 4). However, when only the treatments damaged by *S.exigua* are compared, there were significant difference among microbial treatments in average peak intensities of following six features: F4 ($F(2,12) = 4.274$, $p = 0.0397$ *), F16 ($F(2,12) = 4.98$, $p = 0.0266$*), F17 ($F(2,10) = 8.637$, $p = 0.00663$ **), F19 ($F(2,12) = 6.649$, $p = 0.0114$ *), and F20 ($F(2,10) = 4.366$, $p = 0.0434$ *). Tukey post-hoc tests revealed significantly higher emission of F16, F19, F20, and Sesquiterpene F17 in *T. harzianum* + *S. exigua* and higher emission of F4 in *R. irregularis* + *S. exigua* compared to Non-inoculated + *S. exigua* (data not shown but see Fig. 5).

As shown in Fig. 5 frame A_2, most volatile samples of *S. exigua*-infested leaves are clearly clustered by microbial treatments. Especially, *T. harzianum* + *S. exigua* are distinctly separated from Non-inoculate + *S. exigua* and *R. irregularis* + *S. exigua* due to higher emissions of 11 features in the yellow frame B_1 and lower emissions of the other features in the yellow frame B_2. Even though most of the compounds are not annotated by the NIST library, as shown in Table 2, this result corresponds to the outcome of ANOVA. Most samples of *R. irregularis* + *S. exigua* are clustered separately from Non-inoculate + *S. exigua* samples, as shown in the blue frame C. However, there were no distinct rules in the amount of each volatile feature that contributed to this separation. By contrast, the volatile profiles of non-herbivory treated samples were not distinctly clustered by microbial treatments, as shown in Fig. 5 above the green frame A_1. In summary, microbial inoculation of *R. irregularis* or *T. harzianum* changed the volatile emissions upon *S. exigua* herbivory but not the constitutive volatile emissions.

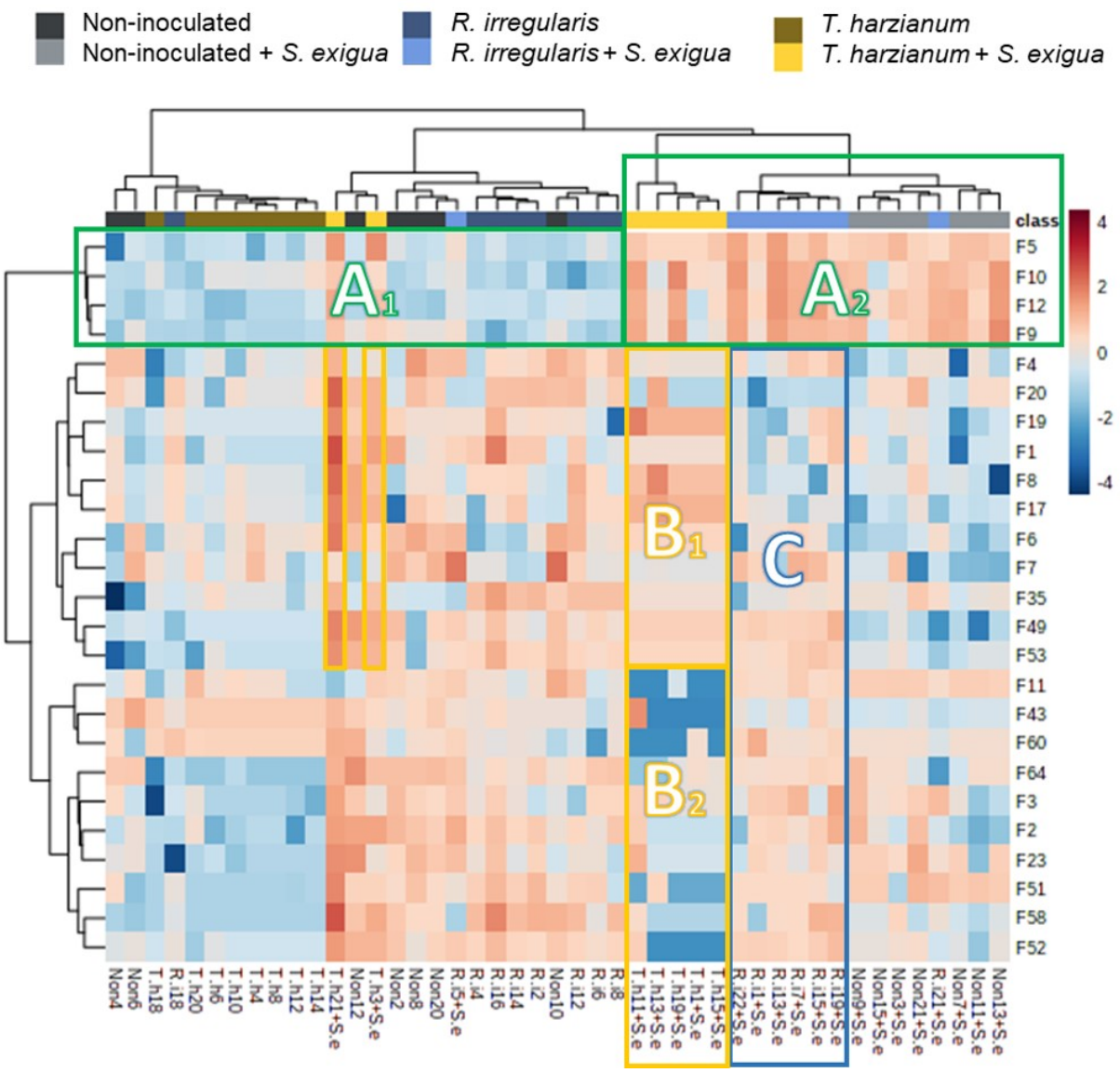

Figure 5 *Clustering Result Visualized as Heatmap*

The heatmap shows the relative peak intensity of the top 25 critical features, illustrated through a chromatic scale: from low (dark blue) to high (dark red). The features marked with the green frames indicate higher peak intensities in A_2, which correlates with the clustering of volatile samples from *S. exigua*-herbivory treatments (A_2) compared to volatile samples from non-herbivory treatments (A_1). The yellow frames show that relatively higher peak intensities of features in B_1 and relatively lower peak intensities of features in B_2 correlate with the clustering of *T. harzianum* + *S.exigua* compared to the other herbivory treated samples. The blue frame (C) indicates no distinct rules between *R. irregularis* + *S. exigua* and Non-inoculated + *S. exigua*. Ward.D is used as a clustering algorithm for dendrogram, and Euclidean is used as distance measure. Missing values (NA: peak intensities lower than the background noise) were replaced by mean values, and log-transformed data were auto-scaled by feature.

4.2 Tomato Indirect Defense Associated Gene Expression

I planned to assess whether microbial symbiosis by *R. irregularis* or *T. harzianum* affects the indirect-defense-gene regulation in response to *S. exigua* herbivory. Using RT-qPCR, I analyzed transcript levels of six indirect-defense related genes extracted from tomato leaf material. The six marker genes are known to be associated with indirect defenses in tomato plants (see text 2.6 and Tab. 1) and categorized into two groups according to the related signaling pathways which were determined by literature research: Jasmonate (JA)-pathway-related defense genes LOX, LOXA, AOS2, and TPS5, and Salicylate(SA) and pathogen defense-related genes PAL and SAMT. Relative fold changes of each gene expression level in tomato plants which are inoculated by *R. irregularis* or *T. harzianum* and infested by *S. exigua* or not, were compared to tomato plants without microbial association and herbivory. The differences in average gene expression levels were analyzed using Two-way ANOVA with Herbivory (H) and Microbe (M) as factors. Regardless of a significant interaction effect, comparisons of treatment means were performed using Tukey multiple comparison test as suggested by Wei et al. (2012)

4.2.1 JA-Associated Gene Expression Levels

I found that the expression levels of four target genes associated with the JA pathway were significantly up-regulated in response to *S. exigua* feeding (See Fig. 6 a-d, $\mathbf{P_H}$***). Although, Two-way ANOVA revealed that there was no significant interaction effect ($\boldsymbol{p_{H\times M}}$ ns) in all JA-associated genes, a statistically significant variation in LOX, LOXA, TPS5 gene expression was explained by microbe (P_M * or **). When *S. exigua* herbivory was absent, microbial colonization resulted in lower gene expression levels of LOXA and TPS5. However, when *S. exigua* fed on tomato plants, differences in transcript levels of JA-associated genes among microbial treatments were not significant (Fig. 6 a-d).

Lipoxygenase gene LOX showed statistically significant differences in average gene expression by both herbivory ($f(1)= 560.435$, $p<0.001$) and microbe ($f(2) = 4.983$, $p<0.05$), though the interaction between these was not significant. A Tukey posthoc test revealed significant pairwise differences by *S. exigua* herbivory (+2.152466 $\log(2^{-\Delta\Delta Ct})$ under *S. exigua* herbivory) and between *T. harzianum*-inoculated and Non-inoculated plants (-0.38352067 $\log(2^{-\Delta\Delta Ct})$ under *T. harzianum*).

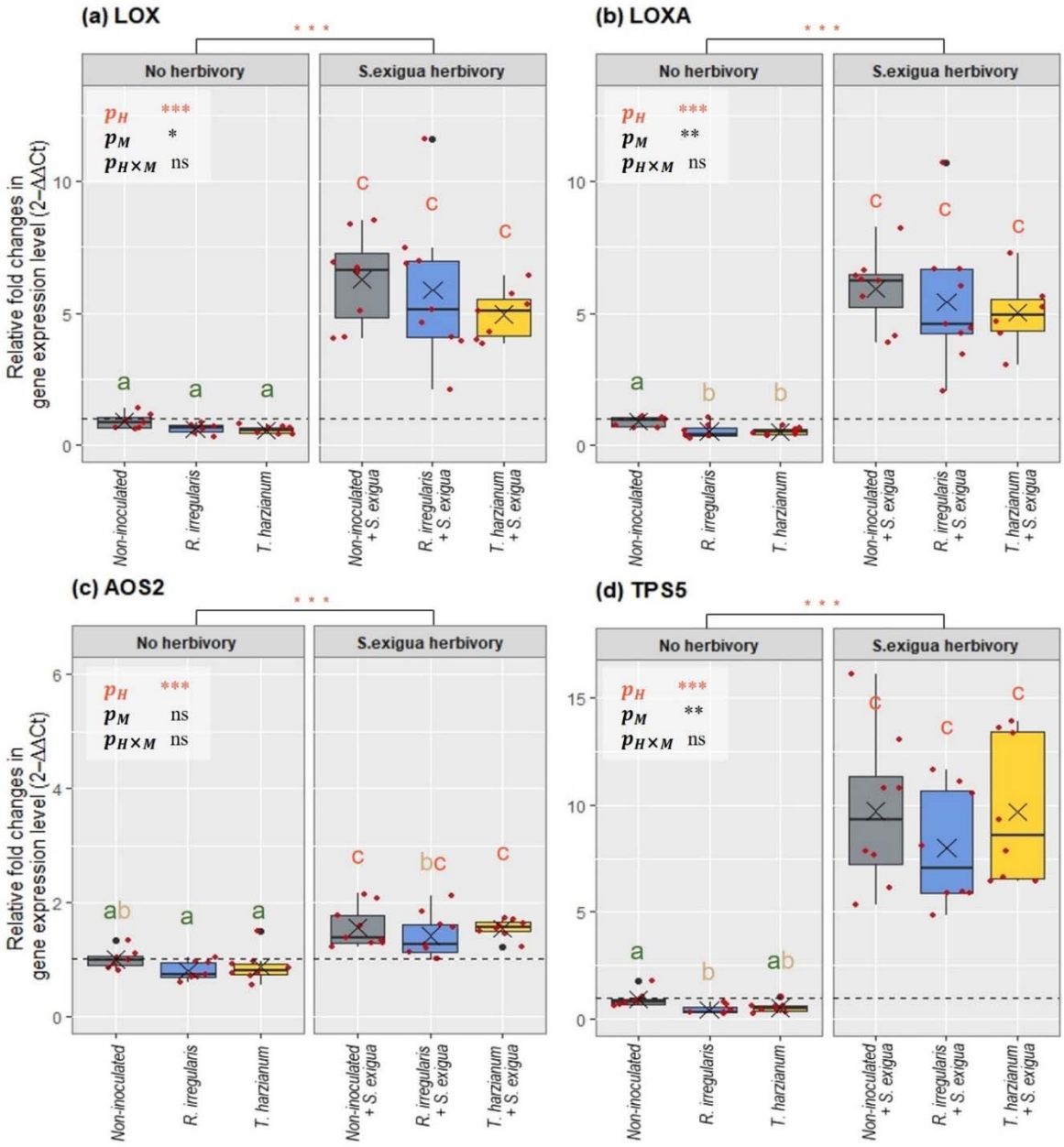

Figure 6 *Relative Gene Expression Levels of JA-Associated Genes*

Target genes are significantly up-regulated ($2^{-\Delta\Delta Ct} > 1$) by *S. exigua* herbivory (a-d) and down-regulated ($2^{-\Delta\Delta Ct} < 1$) by microbial symbiosis when the herbivory is absent (b, by *R. irregularis* or *T. harzianum*; d, by *R. irregularis*). The transcript levels of each gene in tomato plants inoculated with *R. irregularis* or *T. harzianum* and/or infested with *S. exigua* are compared with non-inoculated tomato plants without herbivory (≅ **1**). Only the values indicated with different letters are significantly different (Tukey's HSD test after Two-way ANOVA: $P < 0.05$). P-values of each factor (p_H, the herbivory effect; p_M, the microbial effect) and interaction effect ($p_{H\times M}$) are shown as ns ($p>0.05$), * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$). The mean values are presented with the symbol X and the individual data points are shown in red dots over each boxplot. The gene expression levels in tomato leaves are measured by RT-qPCR (3 technical replicates) and SlEF gene is used for normalization of all target genes. (a-b) LOX and LOXA, lipoxygenase genes; (c) AOS, allene oxide synthase gene; (d) TPS5, (R)-linalool synthase gene.

Another lipoxygenase gene, LOXA showed a statistically significant difference in average gene expression by both herbivory ($f(1)= 490.267$, $p<0.001$) and by microbe ($f(2) = 6.138$, $p<0.01$), though the interaction between these was not significant. A Tukey posthoc test reveals significant pairwise differences by *S. exigua* herbivory (+2.172002 $\log(2^{-\Delta\Delta Ct})$ under *S. exigua* herbivory). And when herbivory is absent, there were significant pairwise differences between *R. irregularis*-inoculated and non-inoculated plants (-0.59401803 $\log(2^{-\Delta\Delta Ct})$ under *R. irregularis*), and between *T. harzianum*-inoculate and non-inoculated plants (-0.55071695 $\log(2^{-\Delta\Delta Ct})$ under *T. harzianum*).

Allene oxide synthase gene, AOS2, revealed statistically significant differences in average gene expression by only herbivory ($f(1)= 66.766$, $p<0.001$). A Tukey posthoc test reveals significant pairwise differences by *S. exigua* herbivory (+0.5225435 $\log(2^{-\Delta\Delta Ct})$ under *S. exigua* herbivory).

The gene coding for (R)-linalool synthase, TPS5 had statistically significant differences in average gene expression by both herbivory ($f(1)= 556.442$, $p<0.001$) and by microbe ($f(2) = 5.652$, $p<0.01$), though the interaction between these was not significant. A Tukey posthoc test reveals significant pairwise differences by *S. exigua* herbivory (+2.816537 $\log(2^{-\Delta\Delta Ct})$ under *S. exigua* herbivory), and between undamaged *R. irregularis*-inoculated and non-inoculated plants (- 0.78706978 $\log(2^{-\Delta\Delta Ct})$ under *R. irregularis*).

4.2.2 SA-Associated Gene Expression Levels

I observed that the expression levels of two target genes associated with the SA pathway were significantly up-regulated in response to *S. exigua* feeding (See Fig. 7 a-b, $\mathbf{P_H}$***). Two-way ANOVA showed no significant interaction effect ($\boldsymbol{p_{H\times M}}$ ns) in PAL, but SAMT (Fig. 7 a-b). Moreover, a significant amount of variation in PAL gene expression is explained by microbe (P_M *). Still, Tukey posthoc tests showed no significant differences among microbial treatments both when plants are infested by *S. exigua* and not.

Phenylalanine ammonia-lyase gene, PAL, had statistically significant differences in average gene expression by both herbivory ($f(1)= 103.947$, $p<0.001$) and by microbe ($f(2) = 3.625$, $p<0.05$). However, the interaction between these was not significant. A Tukey posthoc test revealed significant pairwise differences by *S. exigua* herbivory (+0.8797524 $\log(2^{-\Delta\Delta Ct})$ under *S. exigua* herbivory).

Salicylic acid methyltransferase gene, SAMT showed a statistically significant interaction between herbivory and microbial treatment ($f(2)= 6.590$, $p<0.01$), and a significant herbivory

effect (f(1)= 216.572, p<0.001). A Tukey posthoc test revealed significant pairwise differences by *S. exigua* herbivory (+1.415253 log($2^{-\Delta\Delta Ct}$) under *S. exigua* herbivory). Therefore, the significant interaction effect ($\mathbf{P_{H\times M}}$) indicates that herbivory of *S. exigua* affects the transcript levels of SAMT in microbe-inoculated treatments differently than in non-inoculated plants. Although the differences among microbial treatments infested by *S. exigua* were not significant, herbivory increased SAMT expression levels in *T. harzianum*-inoculated plants (3.11-fold change $2^{-\Delta\Delta Ct}$ difference) to a greater extent than in non-inoculated plants (1.81 fold change$2^{-\Delta\Delta Ct}$ difference; Supplementary material 3 Tab. 7).

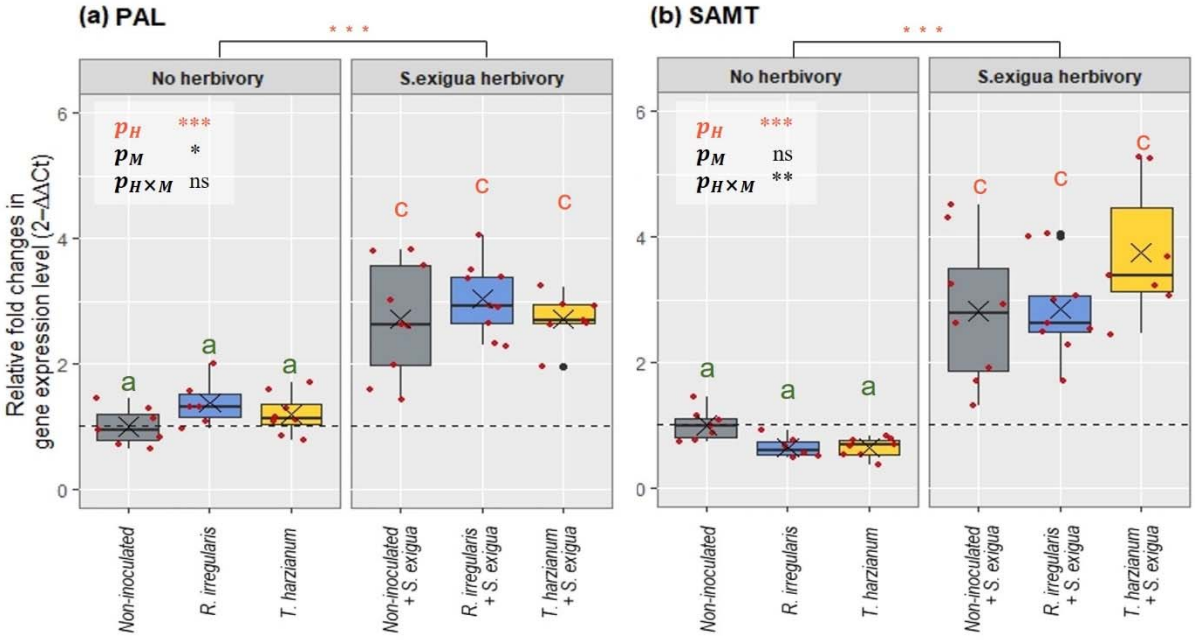

Figure 7 *Relative Gene Expression Levels of SA-Associated Genes*

Target genes are significantly up-regulated ($2^{-\Delta\Delta Ct}$> 1) by *S. exigua* herbivory (e-f) where microbial treatment did not significantly affect. The transcript levels of each genes in tomato plants inoculated with *R. irregularis* or *T. harzianum* and/or infested with *S. exigua* are compared with non-inoculated tomato plants without herbivory ($\cong \mathbf{1}$). Only the values indicated with different letters are significantly different (Tukey's HSD test after two-way ANOVA: P < 0.05). P-values of each factor ($\boldsymbol{p_H}$, the herbivory effect; $\boldsymbol{p_M}$, the microbial effect) and interaction effect ($\boldsymbol{p_{H\times M}}$) are shown as ns (p>0.05), * (p<0.05), ** (p<0.01) and *** (p<0.001). The mean values are presented with the symbol X and the individual data points are shown in red dots over each boxplot. The gene expression levels in tomato leaves are measured by RT-qPCR (3 technical replicates) and SlEF gene is used for normalization of all target genes. (e) PAL, phenylalanine ammonia-lyase gene; (f) SAMT, salicylic acid methyltransferase gene.

4.3 Behavioral Assay Using Y-shape Olfactometer

Lastly, I performed five sets of bioassays using different pairs of odor sources to compare the relative selections made by *M. pygmaeus*. For Tests 1 and 2, I aimed to examine the preference of *M. pygmaeus* towards tomato plant HIPVs in the set olfactometer assay system (see Fig. 3). For Tests 3, 4, and 5, I used the same bioassay system to test the relative preference of *M. pygmaeus* toward HIPVs from microbe-inoculated tomato plants and non-inoculated tomato plants.

4.3.1 Response of *M. pygmaeus* Toward *S.exigua*-induced Tomato Plant Volatiles

Tests 1 and 2 together showed that the Y-tube olfactometer setup used in this study is effective in testing the preference of female adult *M. pygmaeus* (Fig. 8). Test 1 was conducted for the difference in the proportions of selection made by female *M. pygmaeus* preferring tomato plant volatile versus clean air. There is a statistically significant difference in the proportion of *M. pygmaeus* preferring tomato plant volatiles over clean air (X^2(2, N=47) v = 4.79; p <0.05).

Test 2 was performed to analyze the difference in the proportions of choice made by *M. pygmaeus* between *S. exigua*-induced tomato plant volatiles and tomato plant volatiles without herbivory. There is a statistically significant difference in the proportion of *M. pygmaeus* preferring *S. exigua*-induced VOCs over constitutive tomato plant volatiles (X^2 (2, N = 37) v = 11.92; p <0.001).

4.3.2 The Effect of Microbial Symbiosis on the Preference of *M. pygmaeus* Toward HIPVs

From Test 3, 4, and 5, I observed that plants colonized with *R. irregularis* were more attractive than non-inoculated plants, and *T. harzianum*-inoculated plants were more attractive than *R. irregularis* -inoculated plants to female *M. pygmaeus* (Fig. 8). Test 3 was conducted for the difference in the proportions of selection made by *M. pygmaeus* preferring between HIPVs of *R. irregularis*-inoculated plants and non-inoculated plants. There is a statistically significant difference in the proportion of *M. pygmaeus* choosing HIPVs of *R. irregularis*-inoculated plants over tomato plant volatiles without microbial symbiosis (X^2 (2, N = 43) v = 6.72; p <0.01).

Test 4 was implemented for the difference in the proportions of selection made by *M. pygmaeus* preferring between HIPVs of *T. harzianum*-inoculated plants and non-inoculated plants. There is no statistically significant difference in the proportion of *M. pygmaeus* that chose HIPVs of

T. harzianum-inoculated plants over tomato plant volatiles without microbial symbiosis (X^2 (2, N = 47) v = 0.53; p =0.4658).

Test 5 was aimed to evaluate the difference in the proportion of selection made by *M. pygmaeus* preferring between HIPVs of *R. irregularis*-inoculated plants and *T. harzianum*-inoculated plants. There is a statistically significant difference in the proportion of *M. pygmaeus* preferring HIPVs of *T. harzianum* inoculated plants over *R. irregularis* inoculated plants (X^2 (2, N = 43) v = 10.26; p <0.01).

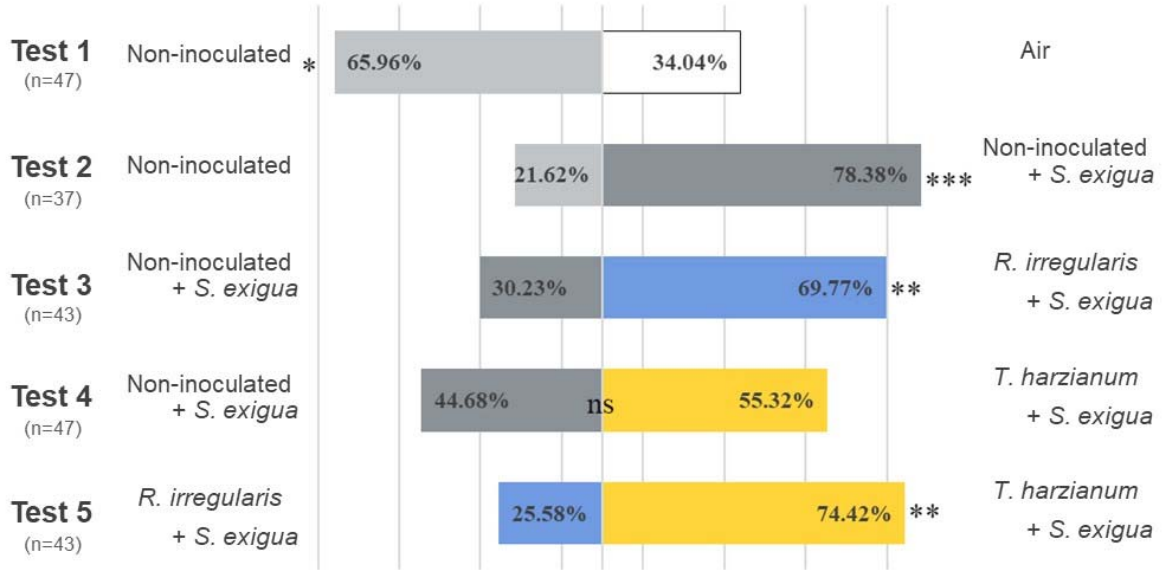

Figure 8 *Comparison of Selection Rates Made by M. pygmaeus in a Y-tube Olfactometer*
Effect of soil symbiosis by *R. irregularis* or *T. harzianum* on the selections made by the generalist predator *M. pygmaeus* in a Y-tube olfactometer (See Fig. 3) was tested. The selection results are presented and written as proportions, and the statistical significance according to Chi-square Test are shown as ns (p>0.05), * (p<0.05), ** (p<0.01) and *** (p<0.001). For each test, five replicates of each odor source set were used and the number of female adult insects who made choices are written (n). Odor sources were provided as following: **Air**, an empty jar; the rest, a jar with a whole tomato plant non- /inoculated by *R. irregularis* or *T. harzianum* without/after 24hour of *S. exigua* larvae herbivory.

5. Discussions and Conclusions

This study provides an expanded understanding of the interaction among soil microbes, polyphagous herbivores, and omnivorous predators through plant defense, which has been poorly discovered. A multidimensional approach was employed using state-of-the-art technologies in volatolomics and transcriptional analyses followed by behavioral assays. As indicated previously, the results show that the plant volatile profiles were changed by herbivory of *S. exigua* and root colonization of *R. irregularis* or *T. harzianum*. Although the quantities of well-known indirect-defense-related volatile compounds and transcriptional levels of JA-/SA-pathway-associated genes were not significantly increased by microbial symbioses, overall changes resulted in a higher preference of *M. pygmaeus* toward microbe-inoculated tomato plants. Together, these results demonstrate the multitrophic interactions across below ground and aboveground mediated by microbe-induced -resistance, which used to be found in a limited range of specialist arthropods. The following sections describe the meanings of the results, accumulated evidence, and implications in greater detail.

5.1 Generalist Chewing Herbivore, *S. exigua* Induces Significant Indirect Defense

I observed from every approach that chewing herbivore *S. exigua* induced distinct volatile emissions including GLVs, and significantly higher transcriptional levels of indirect-defense-related genes. Moreover, omnivorous predator *M. pygmaeus* responded to these changes positively and chose the *S.exigua*-induced plant volatiles.

Herbivory-induced resistance has been reported since the 1970s (Green & Ryan, 1972), and now related metabolites, enzymes are well documented (Schoonhoven et al., 2005), to which my results corresponded. Among detected volatile features, *S. exigua* herbivory induced higher GLV emissions but changes in other indirect-defense-related compounds were not significant. Feeding of *S. exigua* is known to cause higher emission of most GLVs, monoterpenes, several sesquiterpenes, and fatty acids in tomato plants but not every compound was significant (Zebelo et al., 2014). Emissions of indirect-defense-related volatiles are regulated by biosynthetic pathways and enzymes (Koeduka et al., 2020). In general, chewing herbivores are related to JA-pathway, while SA-pathway works against disease and pathogen. Significantly higher expression levels in every targeted JA-related gene and SA-related gene upon *S. exigua* herbivory in my experiment correspond to the results of other researchers (Jafarbeigi et al., 2020; Musser et al., 2012; Rodriguez-Saona et al., 2010; Zebelo et al., 2014). However, the

observed up-regulation of monoterpene synthesis gene TPS5 and MeSA producing gene SAMT was not consistent with measured quantities of these compounds (Tab. 2). Nonetheless, bioassays showed that herbivore-induced volatiles were significantly more attractive to *M. pygmaeus* than constitutive volatiles and air. Among tomato induced-volatiles, a-pinene, b-phellandrene, (E)hex-2-enal, and linalool are known to significantly attract *M. pygmaeus* (De Backer et al., 2017). It used to be tested with thrips and white flies, but the bioassay results showed that *M. pygmaeus* also respond to chewing herbivores. Therefore, it can be assumed that *S. exigua*-infested plant volatiles would contain a higher amount of these attractants. Taken together, the accumulated evidence appears to support the hypothesis that *S. exigua*-herbivory-induced changes in volatiles which are regulated by associated gene expression, contribute to a significantly higher preference of *M. pygmaeus*.

These results show that the passive way of volatile trapping, targeted JA-/SA- associated genes, and the Y-tube olfactometer assay system are robust, in line with previous studies. However, the peaks of complex blends of volatiles were not separated enough to be annotated, and the air of greenhouse had a high concentration of volatiles as background noises. Nevertheless, the quantities of volatiles were well-resolved enough to differentiate non-herbivory-treated and *S. exigua*-herbivory-treated plants by detected features. In conclusion, the employed approaches, biotic systems, and methods were able to demonstrate significant differences in *S. exigua*-damaged plants in all analyses performed. Therefore, they seem to be appropriate tools to investigate the effect of microbial symbioses on the indirect defense response against *S. exigua*.

5.2 Soil Microbial Symbioses Alter the Tomato HIPV Profiles

I observed that under *S. exigua* herbivory, tomato plants volatiles are altered by microbial treatments. Especially *T. harzianum-inoculated + S. exigua* showed significantly higher quantities of several volatile features, including unknown sesquiterpene than non-inoculated plants, corresponding to ANOVA results. Although there were high variations among samples, hierarchical clustering demonstrates that microbial treatments of two different fungi changed HIPVs profiles, which may contribute to changes in interactions with other organisms.

It has been reported that various microorganisms modify VOC-mediated interactions surrounding plants (Dicke & Lucas-Barbosa, 2020) by changing responses at chemical and molecular levels (Pineda et al., 2017). Recent research provided a few case studies on the effect of beneficial-microbe-induced indirect defense against pathogens and insects. Mycorrhizal inoculation resulted in slight changes in VOCs in *S. exigua*-infested *Medicago truncatula*

(Leitner et al., 2010) and spider-mite-infested bean plants (Schausberger et al., 2012). And mycorrhiza-inoculated poplars showed unique VOC patterns and up-regulation of genes for monoterpene and sesquiterpene emission when herbivory was absent (Kaling et al., 2018). When *T. harzianum* was inoculated in a maize field, the concentrations of certain VOCs increased, and subsequently, the number of pest-regulating arthropods significantly rose (Contreras-Cornejo et al., 2020). Similar results were shown by *T. harzianum*-inoculated tomato upon aphid attack (Coppola et al., 2017). Tomato plants inoculated with *T.atroviride*-showed *ex novo* production of z-3-hexenol, d-2-carene, limonene, and methyl salicylate, and significantly increased emissions of a-pinene and b-cymene (Coppola et al., 2019).

Unfortunately, previously reported volatile compounds were statistically insignificant or not annotated in this study. Therefore, further research is required to employ standard compounds in the volatile trapping and analysis process to identify which volatile compounds are significantly changed by microbial colonization in plants. Moreover, there were higher variations among volatile samples in microbe-inoculated and *S. exigua*-damaged plants than non-inoculated plants. This result implies that it is necessary to evaluate whether microbial symbionts affected the leaf damage rates by exploiting direct defense (Koricheva et al., 2009) and if microbial colonization rates and stages contributed to the observed variations. Nevertheless, this experiment investigated the respective effect of two different soil microbes on plant volatiles against generalist chewing herbivores, expanding the microbe-species-specific tendency (Pineda et al., 2017) and knowledge mostly limited to most specialist pests with single microbes.

In conclusion, although most compounds are unknown, root symbioses induced changes in VOC profiles upon aboveground herbivory. The robust clustering made by microbial treatments implies surrounding organisms may perceive these changes as a cue, which may induce modified indirect defense of plants. When the beneficial effect of microbial symbionts in plant defense is confirmed, used microbes can replace not only fertilizers for growth-promoting but also chemical pesticides in sustainable agricultural practices.

5.3 Microbial Symbioses Affect Regulation of Tomato Indirect Defense Genes

In this study, I observed that microbial symbioses affect some of JA-/SA- related gene expression, but the differences were not statistically significant after *S. exigua* herbivory for 24hr. When not infested by the herbivore, I found that both microbes down-regulated LOXA

gene encoding for JA and further GLV production and *R. irregularis* downregulated TPS5 gene encoding for monoterpene Linalool synthesis. This result shows that symbioses suppressed some indirect-defense-related signaling. In addition, microbe-inoculated plants did not respond to herbivory to a higher extend except for SAMT. A significant interaction effect found in SAMT showed that, in response to *S. exigua* herbivory, *T. harzianum* induced the plants to synthesize MeSA to a greater extent than in *R. irregularis*-inoculated and non-inoculated plants.

Symbioses with obligate biotrophs AMF are generally known to suppress the SA-dependent defense in plants to establish symbiotic status, which increases antagonistic JA response levels associated with resistance to necrotrophic pathogens and chewing herbivores (Bonfante & Genre, 2015; Pozo & Azcón-Aguilar, 2007). Although variations were observed, up-regulated JA genes by AMF would enable plants to respond stronger and faster (Kaling et al., 2018; Koricheva et al., 2009). Stronger responses in JA-pathway associated genes (Pineda et al., 2010; Pozo & Azcón-Aguilar, 2007; Song et al., 2013) and, to a lesser extent, in SA- and ET-pathways are the most common molecular responses which are reported in AMF-Solanaceaea-chewing insect interactions (Gruden et al., 2020). Together they suggest that AMF colonization primes plant defense, and several pathways are involved.

However, Mycorrhizal inoculation itself does not always induce transcript changes (Song et al., 2013), as Pieterse et al. (2000) suggested that ISR is based more on sensitivity to phytohormones rather than on the production. In this study, I could not observe a higher response in JA biosynthesis and JA responsive genes in *R. irregularis*-inoculated plants upon *S. exigua* herbivory. In contrast, the reaction of SA synthesis gene PAL was slightly increased by *R. irregularis* colonization. These results may attribute to the incomplete establishment of mycorrhizas (Pozo & Azcón-Aguilar, 2007), and the stage of symbiotic reprogramming was still underway four weeks after inoculation. The lower expression levels of JA-related genes LOXA and TPS5 in *R. irregularis*-inoculate plants maybe because the interaction between JA-signaling pathways with SA and other phytochrome pathways attenuates the resistance of plants (Thaler, Fidanstef and Bostock, 2002). It had been reported that AMF inoculation increased the concentration of phytohormone GA, ZR, and JA and changed ABA, IAA, GA, ZR, and the JA content upon *S. exigua* attack (He et al., 2017). Furthermore, the symbiotic association may engage in plant growth promotion for nutritional exchange rather than induced resistance when herbivory and pathogen are absent (Pineda et al., 2010; Pozo & Azcón-Aguilar, 2007).

Plant growth-promoting fungi *Trichoderma* spp. can change plant hormonal homeostasis by producing secretion and volatiles, including hormones and enzymes. Along the way, *Trichoderma* spp. interfere with plant physiology, and promote plant growth (Alfiky &

Weisskopf, 2021). These changes cascade to the defense response. In this way, *Trichoderma* spp. are known to stimulate ISR in plants against various insect herbivores (Coppola et al., 2019; Jafarbeigi et al., 2020; Muvea et al., 2014) by modulating JA, ET, and SA dependent defense (Pieterse et al., 2014)

It has been reported that *Trichoderma* spp. colonization can increase JA-signaling-related gene responses, including LOX, AOS, TPSs, and ET biosynthesis (Alfiky & Weisskopf, 2021; Cai et al., 2013; Coppola et al., 2019; Di Lelio et al., 2021). However, increased SA production and expression of several SA-marker genes and pathogenesis-related genes upon herbivory attack were also reported (Adss et al., 2021; Malmierca et al., 2015a; Malmierca et al., 2015b; Martínez-Medina et al., 2017b). These findings are consistent with the increased SAMT expression in my experiment. Likewise, Coppola et al. (2017) reported downregulation of PAL in *T. harzianum*-colonized and aphid-infested tomato while upregulations in SAMT and LOX. This non-uniform pattern of JA- and SA-associated gene expression under attack reveals the possible crosstalk among these signaling pathways, especially between JA and SA (Martínez-Medina et al., 2017a; Pieterse et al., 2014). Moreover, a plastic phenomenon in ISR was reported, where *T. harzianum* adapted JA- and SA-dependent defense according to the stage of nematode infection (Martínez-Medina et al., 2017a). Therefore, it can be suggested that the presence and alternative activation of transduction pathways involved may allow the inoculated plants to react to a broad spectrum of pests and diseases.

In this study, I investigated a few JA-and SA-signaling-related genes. However, ET and other phytohormones such as ABA, auxins, GA, and CK are also known to affect this JA-SA backbone by synergizing or antagonizing one of them (Morán-Diez et al., 2020; Pieterse et al., 2012; Wang et al., 2019), also depending on the inoculated *Trichoderma* species (Di Lelio et al., 2021). Therefore, a transcriptomic approach and quantification of these phytohormone contents could provide a complete snapshot of overall changes in plant signaling pathways. In addition, because the phytohormone modules in multiple interactions are fine-tuned in timing and strength (Gruden et al., 2020), the temporal aspect should be considered. Peng et al. (2004) observed that the transcription of PR1, BGL2, and PAL was strongly induced 1 hr after herbivory and kept increasing only until 6 hr but began to decrease after 6 hr. My experiment sampled damaged leaf material 5 hours after *S. exigua*-herbivory of 24 hours. Therefore, it will be possible to explain the dynamic changes in the network properties in signal pathways by sampling several time points. Lastly, it should be considered that abiotic factors may have interacted with the microbe-induced resistance in plants. High soil P concentration inhibits the perception of JA by AMF and enhances other antagonistic hormonal compositions (Bedini et

al., 2018). In the work of Qu et al. (2021), high soil P and light availability repressed JA-dependent defense and rendered the AMF-induced-resistance ineffective in young plants. In addition, the levels of resistance induced by two *Trichoderma* species were temperature-dependent (Di Lelio et al., 2021).

In summary, the results demonstrated that the association with beneficial soil fungi does not always induce a higher transcriptional response in both JA and SA signaling upon caterpillar feeding. However, downregulated JA-related genes in microbe-inoculated plants and significant microbe and herbivory interaction effects in SAMT expression indicate that microbial symbioses modify the activities of these defense-related signaling. Priming plants for rapid and robust defense response against insect pests and pathogens is vital for crop plant growth and yield. However, whether beneficial microbes induce higher plant resistance in different conditions remains unclear according to this and other studies. Nevertheless, there is a strong possibility that our continuously growing knowledge of multiple signal transduction pathways in plants would enable sustainable crop plant protection.

5.4 Effect of Microbial Inoculation on the Response of *M. pygmaeus*

In this study, I observed that *M. pygmaeus* positively responded to microbe-modified VOCs upon *S. exigua* feeding. A statistically significant number of plant bugs preferred volatiles of *R. irregularis* inoculated plants than non-inoculated plants. Relatively weaker preference to volatiles of *T. harzianum* inoculated plants than non-inoculated plants is presumed to attribute to the high variations in volatile profiles of *T. harzianum* inoculated plants in response to herbivory (See yellow triangles in Fig. 4). Nevertheless, the symbiosis with *T. harzianum* successfully attracted a significantly higher number of predators compared to *R. irregularis*. This demonstrates that underground microbial symbioses can contribute to the higher attraction of generalist predators when plants are attacked by polyphagous chewing herbivores aboveground.

When the performance of herbivore enemies is positively affected, it is called indirect resistance (Schoonhoven et al., 2005). A few studies have investigated the attraction of predatory mirid bugs toward plant volatiles and synthesized compounds. Mirid bug *M. pygmaeus* distinguished VOCs of tomato plants infested by leaf miner (De Backer et al., 2015a) or peach aphid (De Backer et al., 2015b) from non-infested plants. Close plant bug *Macrolophus caliginosus* responded to volatiles of several crop plants infested by whitefly (Saad, Roff, Salam, & Idris, 2014), two-spotted spider mite (Moayeri et al., 2007), and infested by spider mites and aphids

simultaneously (Moayeri et al., 2007). Considering that the omnivorous mirid bugs are one of the natural enemies of lepidopteran eggs and young larvae, *M. pygmaeus* may respond to volatiles of plants attacked by *S. exigua*.

An increasing number of studies are investigating the effect of beneficial microbe on the behavior, density, reproduction, and parasitism rates of natural enemies. Mycorrhizae are reported to affect the herbivore enemies' preference (Guerrieri et al., 2004; Hoffmann et al., 2011a; Schausberger et al., 2012), density (Gange et al., 2003; Schreck et al., 2013), and developmental and reproductive traits (Hempel et al., 2009; Hoffmann et al., 2011b; Moon et al., 2013). In particular, AMF increased the attraction (Schausberger et al., 2012) and oviposition rate of predatory mites (Hoffmann et al., 2011a), and aphid parasitism rate (Hempel et al., 2009). Similarly, *Trichoderma* spp. significantly increased the attraction of aphid-parasitic wasp (Battaglia et al., 2013; Coppola et al., 2019), and changes in leaf metabolites after *T. harzianum* inoculation increased pest regulating arthropods in a maize field (Contreras-Cornejo et al., 2020). In contrast, several field experiments did not find a positive effect of AMF on the herbivore density (Schreck et al., 2013) and the abundance of generalist predator *Orius sauteri* (Ueda et al., 2013). Another important finding was that several species of AMF and their combinations caused differences in plant size (Gange et al., 2003) and increased the leaf number (Moon et al., 2013), which affected the searching efficiency of parasitoids and resulted in lower parasitism rates. Rasmann et al. (2017) pointed out that besides modified plant metabolites, plant vigor and microbial VOCs from the soil can also change the fate of indirect defense performance. Moreover, a meta-analytic study analyzed that the effects of microbial mutualism on natural enemies were inconsistent and highly differed depending on the measured parameter and introduced fungal species (Tao et al., 2017).

As Stenberg (2017) described, the biological control *M. pygmaeus* agent sensitively reacted to the changes in plants made by plant vaccination elements, microbial inoculation. The results of my bioassays give a broad hint that microbial symbioses positively affect the behavioral response of omnivorous predators to the plant defense cue against generalist chewing herbivores. This biological system has not gotten attention due to the low level of specialization in the connection of organisms; however, it can be utilized in the standard greenhouse agricultural practices for IPM. However, because the efficacy of the pest control was not measured, and therefore, it is early to confirm the microbe-induced resistance. The density of the targeted pathogen or pest is the critical determinant to evaluate the effectiveness of ISR (Lee Díaz et al., 2021), and the reshaped multi-trophic community structure may alter the nutrient and energy cycling of the whole ecosystem (Ferlian et al., 2018). Therefore, researchers should

evaluate the changes in the pest population, enemy reproduction rates, and the long-term effect on our agricultural ecosystem.

5.5 Conclusion and Perspective

The results of bioassays in this study show that symbioses with soil microbes can render *S. exigua*-induced plant volatiles more attractive to the generalist predator *M. pygmaeus*. Although the results of the greenhouse experiment followed by volatile and transcript analyses did not show statistically significant quantitative differences, this discrepancy demonstrates the context-dependency of ISR. To deal with the high context dependency and to optimize the exploitation of microbe-induced-resistance, the age and developmental stage of the interacting organisms, plant growth conditions, and time-course experiments should be considered (Gruden et al., 2020; Lee Díaz et al., 2021).

In conclusion, I found that symbiotic fungi in the soil influence the plant response to herbivores and the aboveground interactions with herbivore enemies, which may enhance the indirect defense against multiple pests. Considering that *S. exigua* is a non-native pest species in most countries globally and has already developed high resistance to insecticides (Caccia et al., 2014), generalist enemies of this pest are expected to control this pest insect (Darsouei et al., 2018). Because the global changes will bring more invasive pests to conventional agriculture (Singh & Singh, 2017), knowing if current ecological pest management measures would work against these pests and be used instead of chemical pesticides is essential. The results of this study expanded our knowledge on the interaction between commonly used beneficial fungi and omnivorous biological control agents, which will ultimately contribute to reducing pesticides and securing biodiversity.

6. Summary

Careful integration of multiple pest management measures (IPM) is expected to reduce the harmful influence of pesticides and help to secure our biodiversity. It is important to understand plant defense mechanisms and interactions among surrounding organisms through plant metabolomic changes to achieve IPM. Especially whether commonly used symbiotic root fungi can raise Induced-Systematic-Resistance (ISR) is taking center stage, but our knowledge has been limited to only a few specialist herbivores and enemies. Therefore, my study aimed to investigate the changes in volatile emission and transcripts of tomato plants infested with *S. exigua* and focused on the effect of two root-symbiotic fungi, *R. irregularis* and *T. harzianum,* on the induction of indirect defense. In addition, to evaluate the effect of these changes on predator attraction, I conducted olfactometer bioassays with *M. pygmaeus*, an omnivorous predator of numerous pest insects.

For a greenhouse experiment, I grew tomato seedlings inoculated with commercial *R. irregularis* inoculum or laboratory strain *T. harzianum* T-78 and non-inoculated seedlings for four weeks. After challenging the plants with *S. exigua* 3rd instar larvae for 24 hours, I trapped leaf volatiles using silicon tubes and collected leaves for RNA extraction. Subsequently, volatile profiles were analyzed using GC-MS, and JA-/SA-related-gene expression was measured using RT-qPCR. In addition, I let female *M. pygmaeus* choose between two odor sources through a Y-shaped tube and analyzed the preference toward HIPVs of microbe-inoculated tomato plants.

Clustering analysis revealed that microbial symbioses resulted in distinct changes in volatile profiles after *S. exigua* herbivory. By contrast, differential gene expression analyses showed suppression by both symbionts in some JA-associated genes, while the SAMT gene showed slight ISR upon herbivory in *T. harzianum*-inoculated plants. The selection made by *M. pygmaeus* was significantly higher in *R. irregularis*-inoculated plant HIPVs than in non-inoculated plants, and *T. harzianum*-inoculated plant was more attractive to predators than *R. irregularis*-inoculated plant. These results jointly indicate that the microbial symbionts may induce higher indirect defense by modulating phytohormone signal transduction and volatile emission. The discrepancy in the results and the context-dependency of ISR call for transcriptomic approaches and time-course experiments with careful consideration of abiotic factors. Finally, this study discovered unnoticed interaction among generalist insects modulated by symbiotic microbes through plant defense and suggested possible integration of current plant protection measures.

7. Zusammenfassung

Durch die sorgfältige Integration mehrerer Pest-Management-Maßnahmen (IPM) soll der schädliche Einfluss von Pestiziden verringert und die Biodiversität gesichert werden. Es ist wichtig, die Abwehrmechanismen der Pflanzen zu verstehen, um nachhaltige IPM zu erreichen. Darüber die durch metabolomische Veränderungen beeinflussten Interaktionen der Pflanzen mit umgebenen Organismen sind dabei von besonderer Bedeutung. Im Mittelpunkt steht dabei, ob die häufig verwendeten symbiotischen Wurzelpilze die Induzierte-Systematische-Resistenz (ISR) erhöhen können. Das bisherige Wissen beschränkt sich allerdings auf wenige spezialisierte Pflanzenfresser und deren Feinde. Daher zielte meine Studie darauf ab, die Veränderungen der flüchtigen Blattduftstoffe und Transkripte von Tomatenpflanzen zu untersuchen, die mit *S. exigua* befallen sind. Die Studie konzentrierte sich auf die Wirkung zweier wurzelsymbiotischer Pilze, *R. irregulăris* und *T. harzianum*, auf die Induktion der indirekten Abwehr. Um die Wirkung dieser Veränderungen auf die Anziehung von Insektenfressern zu bewerten, führte ich außerdem Olfaktometer-Bioassays mit *M. pygmaeus* durch, einem allesfressenden Insektenfressern zahlreicher Schadinsekten.

Für ein Gewächshausexperiment züchtete ich Tomatensetzlinge, die mit kommerziellem *R. irregulăris*-Inoculum oder dem Laborstamm *T. harzianum* T-78 beimpft wurden, und nicht beimpfte Setzlinge für vier Wochen. Nachdem ich die Pflanzen 24 Stunden lang mit Larven von *S. exigua* im 3. Larvenstadium herausgefordert hatte, fing ich flüchtige Blattduftstoffe mit Silikonröhrchen ein und sammelte Blätter für die RNA-Extraktion. Anschließend wurden Profile von den flüchtigen Blattbestandteilen mittels GC-MS analysiert und die JA-/SA-bezogene Genexpression mittels RT-qPCR gemessen. Darüber hinaus ließ ich weibliche *M. pygmaeus* durch ein Y-förmiges Röhrchen zwischen zwei Geruchsquellen wählen und analysierte die Bevorzugung von HIPVs von mit Mikroben inokulierten Tomatenpflanzen.

Clustering-Analysen ergaben, dass mikrobielle Symbiosen zu deutlichen Veränderungen der Profile von den flüchtigen Blattbestandteilen nach *S. exigua*-Herbivoren führten. Im Gegensatz dazu zeigten differentielle Genexpressionsanalysen eine Suppression durch beide Symbionten in einigen JA-assoziierten Genen, während das SAMT-Gen eine leichte ISR in mit *T. harzianum* inokulierten Pflanzen bei Herbivorie zeigte. Die von *M. pygmaeus* getroffene Auswahl war bei mit *R. irregulăris* beimpften Pflanzen-HIPVs signifikant höher als bei nicht beimpften Pflanzen, und mit *T. harzianum* beimpfte Pflanzen waren für Insektenfressern attraktiver als mit *R. irregulăris* beimpfte Pflanzen. Diese Ergebnisse weisen gemeinsam darauf hin, dass die mikrobiellen Symbionten eine höhere indirekte Abwehr induzieren können, indem sie die Phytohormon-Signaltransduktion und die flüchtige Emission modulieren. Die

Diskrepanz in den Ergebnissen und die Kontextabhängigkeit der ISR erfordern transkriptomische Ansätze und Zeitverlaufsexperimente unter sorgfältiger Berücksichtigung abiotischer Faktoren. Schließlich entdeckte diese Studie unbemerkte Interaktionen zwischen generalistischen Insekten, die durch symbiotische Mikroben durch Pflanzenabwehr moduliert werden, und schlug eine mögliche Integration aktueller Pflanzenschutzmaßnahmen vor.

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Supplementary material 1

The following conditions were used for VOC desorption with a flow path temperature of 160 °C: dry purge 5 min at 20 ml/min, pre-purge 1 min at 10 ml/min to remove remaining water, desorption 8 min at 200 °C with 60 ml/min, trap temperature 0 °C, pre trap fire purge 1 min at 60 ml/min, split flow 20 ml/min, trap heated to 230 °C and hold for 4 min. The temperature program of GC was set to the following: 60°C (hold 1 min), 30 °C/min to 150 °C, 10 °C/min to 200 °C and 30 °C/min to 230 °C (hold 1 min). MS conditions were set at 40 °C for the manifold, 240 °C at the transfer line, and 220 °C for the ion source. The scan range was 33–500 m/z for a full scan, and the scan time was 250 ms (Sam et al., 2021).

Table 3 *Parameters used for feature detection in XCMS online*

Menu	Details
General	Polarity : positive Retention time format : minutes
Feature Detection	Method: matched Filter ppm : 200 minimum peak width : 3.5 maximum peak width : 11 mzdiff : 0.1 Signal/Noise threshold : 50 Integration method : 2 prefilter peaks : 3 prefilter intensity : 100000 Noise Filter : 0
Retention Time Correction	Method : obiwarp profStep : 1 step size
Alignment	bw : 1 minfrac : 0.1 mzwid : 0.25 minsamp : 1 max : 100
Annotation	Ppm : 100 m/z absolute error : 0.25 Search for : isotopes
Identification	ppm : 100 adducts : [M+H]+ pathway ppm deviation : 5
Visualization	EIC width : 100

Supplementary Material 2

Following protocols are modified from laboratory protocols of the Molecular Interaction Ecology group led by Professor Nicole van Dam at iDiv in Leipzig, Germany.

Table 4 *Laboratory Protocol for RNA Extraction*

Steps	Material / Reagent	treatment
1	Fresh grind plant material around 100 mg Add 400 µl Cell lysis solution	Homogenize by vortexing and incubate at RT for 5 min on a rotator (program U2)
2	Add 150 µl pre-cooled Protein-DNA precipitation solution	Invert 5 times, incubate on ice for 10 min, then 15min centrifugation 15000rpm 4°C
3	Transfer supernatant to a new tube	5min centrifugation 15000rpm 4°C
4	Transfer supernatant 300 µl to a new tube Add 200 µl pre-cooled Isopropanol	Homogenize by inverting 5 times and 8 min centrifugation 15000 rpm 4°C, and discard the supernatant
5	Add 300 µl 70% Ethanol	Invert 3 times, 8 min centrifugation 15000 rpm 4°C, and discard the supernatant
6	Add 300 µl 70% Ethanol	Incubate 5 min at RT, 5min centrifugation 15000 rpm 4°C, pipette off all the supernatant, and air dry the pellet for 5 min under hood
7	Resuspend pellet in 35 µl Milli pure water (55-60°C)	Determine the concentration with Nanophotometer (LID 50) and store at -80°C

Table 5 *Laboratory Protocol for Quality Check RNA*

Steps	Material / Reagent	Treatment
1	Dilute 5.0 µg total RNA with water up to 34 µl	
2	Prepare a Mastermix : 2 µl 10x DNase I buffer, 1 µl DNase I	Add 3 µl of Mastermix to each sample, mix by pipetting, and incubate 30 min at 37°C
3	Add 1 µl 40mM EDTA	Mix by pipetting and incubate 10min at 65°C
4		Determine the concentration with Nanophotometer (LID 10) and store at -80°C

Table 6 *Laboratory Protocol for cDNA Synthesis*

Steps	Material / Reagent	Treatment
1	Dilute 5.0 µg clean RNA with RNASE free water up to 24 µl	
2	Add 1 µl 50 µM Oligo dT 20	Spin down, incubate for 5 min 65°C, place samples on ice, and spin down again
3	Prepare Master mix: 4 µl 5x RT Buffer, 2 µl 10mM dNTP Mix, and 1 µl RevertAid H Minus Reverse Transcriptase Add 7 µl of Master mix to each sample	Spin down, incubate for 60 min 42°C, 15 min 50°C, 15min 70°C
4		Spin down and store at -20°C

Supplementary material 3

Table 7 *Relative gene expression levels ($2^{-\Delta\Delta Ct}$) of each treatment. The number of biological control used is shown as n under mean ± standard deviation.*

Target gene	Non-inoculated	*R. irregularis*	*T. harzianum*	Non-inoculated + *S. exigua*	*R. irregularis* + *S. exigua*	*T. harzianum* + *S. exigua*
PAL	0.99 ± 0.11 (n=7)	1.37 ± 0.15 (n=6)	1.19 ± 0.11 (n=8)	2.71 ± 0.3 (n=9)	3.04 ± 0.19 (n=9)	2.71 ± 0.14 (n=7)
SAMT	1 ± 0.09 (n=7)	0.64 ± 0.06 (n=6)	0.64 ± 0.05 (n=8)	2.81 ± 0.41 (n=8)	2.85 ± 0.25 (n=9)	3.75 ± 0.41 (n=7)
LOX	0.88 ± 0.11 (n=7)	0.6 ± 0.07 (n=6)	0.55 ± 0.04 (n=8)	6.28 ± 0.61 (n=8)	5.86 ± 0.91 (n=9)	4.95 ± 0.36 (n=7)
LOXA	0.87 ± 0.07 (n=7)	0.52 ± 0.1 (n=7)	0.5 ± 0.04 (n=8)	5.93 ± 0.49 (n=8)	5.42 ± 0.83 (n=9)	5.01 ± 0.58 (n=6)
AOS5	1 ± 0.06 (n=7)	0.8 ± 0.06 (n=7)	0.87 ± 0.09 (n=8)	1.56 ± 0.11 (n=9)	1.4 ± 0.12 (n=9)	1.54 ± 0.05 (n=8)
TPS5	0.91 ± 0.14 (n=7)	0.43 ± 0.08 (n=7)	0.52 ± 0.09 (n=8)	9.71 ± 1.29 (n=8)	7.99 ± 0.97 (n=8)	9.69 ± 1.2 (n=8)

Declaration of Self-Dependence

Herewith I declare that I prepared this thesis on my own, that I did not use any other sources and resources than those that are specified, that all arguments and ideas that were literally or analogously taken from other sources are sufficiently identified, and that the thesis in identical or similar form has not been use as part of an earlier course achievement or examination procedure.

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Place, Date Signature