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How multiple-pest attack impacts plant-mediated indirect interactions on tomato crops?

Yanyan Qu

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Yanyan Qu. How multiple-pest attack impacts plant-mediated indirect interactions on tomato crops?. *Vegetal Biology*. COMUE Université Côte d'Azur (2015 - 2019), 2019. English. NNT : 2019AZUR6035 . tel-03049732

HAL Id: tel-03049732

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THÈSE DE DOCTORAT

Interactions indirectes médiées par la plante sous contraintes biotiques multiples

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Ecologie des Communautés dans les Agro-écosystèmes
UMR INRA 1355

**Présentée en vue de l'obtention
du grade de docteur en** Sciences de la
Vie et de la Santé (ED85)
d'Université Côte d'Azur
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Soutenue le : 9 Décembre 2019

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Interactions indirectes médiées par la plante sous contraintes biotiques multiples

Les plantes, en tant que producteurs primaires, peuvent être consommées/attaquées par de nombreux organismes, notamment des virus, des pathogènes, des insectes herbivores ou encore des nématodes phytophages. D'une part, les plantes sont une ressource alimentaire principale pour ces organismes ; d'autre part, les plantes peuvent se défendre contre ces consommateurs en mobilisant des défenses constitutives et/ou induites. En cas de défenses induites suite à l'attaque d'un ravageur, des interactions indirectes médiées par la plante peuvent apparaître entre ce ravageur et d'autres ravageurs partageant le même hôte *i.e.* la présence d'un ravageur va provoquer des modifications au niveau de la plante qui peuvent à leur tour impacter les autres ravageurs présents sur la même plante.

Tout d'abord, les effets sublétaux de la bêta-cyperméthrine, un pesticide de type pyréthrinoides, ont été évalués sur les interactions interspécifiques entre le puceron spécialiste *Aphis glycines* et le puceron généraliste *Aulacorthum solani* sur soja. La concentration sublétale (LC₅) de bêta-cyperméthrine accélère la compétition interspécifique entre les deux espèces de pucerons. La sensibilité des plants de soja aux pucerons induite par une pré-infestation d'une autre espèce de puceron pourrait ainsi être considérablement réduite par une exposition préalable à la bêta-cyperméthrine sublétaux.

Dans un deuxième temps, nous nous sommes intéressés aux facteurs biotiques susceptibles de moduler les interactions indirectes. Dans ce cadre, divers ravageurs, à savoir le puceron, la chenille, le phytopathogène et le nématode, ont été introduits et suivis sur des plants de tomate, *Solanum lycopersicum*. La performance des ravageurs arthropodes, (e.g. le nombre d'individus de pucerons et le taux de développement des larves de lépidoptères) était corrélée au nombre d'interactions indirectes (*i.e.* au nombre d'espèces de ravageurs partageant le même hôte), que les ravageurs attaquent les plants de tomate séquentiellement ou simultanément. En effet, lorsque les ravageurs étaient mis en présence d'un plant de tomate de manière simultanée, la performance des insectes était positivement corrélée au nombre d'interactions indirectes. A l'inverse, lorsque les ravageurs étaient mis en présence de plants de tomate de façon séquentielle, la performance des insectes ravageurs était négativement corrélée à l'abondance des interactions indirectes. Enfin, aucune corrélation n'a pas été détectée pour les pathogènes, champignons et nematodes.

D'autre part, nous avons pu mettre en évidence que la présence de nématodes et d'oïdium réduisait à court-terme *i.e.* à l'échelle d'une génération l'abondance de pucerons sur un plant de tomate attaqués par ces trois ravageurs. Nous avons voulu tester cette interaction indirecte sur du long-terme *i.e.* à l'échelle d'un suivi de la dynamique de populations des pucerons. De plus, nous avons ajouté à cette expérimentation des parasitoïdes du puceron afin de voir l'impact de ces interactions indirectes sur le troisième niveau trophique. La population de pucerons impliquée dans les interactions indirectes testées a été réduite à court et à long terme parce-que la survie des nymphes diminuait. De plus, le nombre de pucerons ailés a augmenté. Enfin, le taux de parasitisme de l'ennemi naturel *Aphidius ervi*.

Ainsi, ce travail démontre la présence d'interactions indirectes fortes entre les différents ravageurs partageant une même plante hôte et ces interactions sont susceptibles d'être modulées par de nombreux facteurs comme le nombre ou encore la diversité des ravageurs. De plus, elles sont susceptibles de modifier le service de biocontrôle rendu par les auxiliaires des cultures ainsi que le rendement de la culture. Il est donc nécessaire de comprendre et prendre en compte ces interactions indirectes dans le cadre d'attaques multiples pour adopter les stratégies IPM les plus adéquates.

Mots clés: interaction trophique, relation hôte-parasite, interactions plantes-insectes, défense chimique des plantes, biodiversité des ravageurs, insectes herbivores, phytopathogènes

How multiple-pest attack impacts plant-mediated indirect interactions on tomato crops?

In the agroecosystem, plant crops are the primary trophic producer that can be consumed/parasitized by a plethora of organisms, including viruses, bacteria, fungi, nematodes and many herbivore insects. On one hand, plants support these organisms as food resources; on the other hand, plants defend themselves against those consumers by employing constitutive and induced defenses. Both features might be involved in plant-mediated indirect interactions when multiple pests share the same host plant. It means that the presence of one pest might have an indirect effect on another plant through the modification of some plant host features (nutritional quality or defense).

During my Ph.D. study, I assessed the modulating factors (both abiotic and biotic factors) that might affect the plant-mediated indirect interactions, and also investigated the influences of such indirect interactions at long term and on a tri-trophic system, and finally analyzed the underlying phytochemical mechanisms by metabolomics analyses.

Firstly, sublethal effects of beta-cypermethrin, a pyrethroid pesticide as the abiotic factor, were assessed on the interspecific interactions between the specialist aphid *Aphis glycines*, and the generalist aphid *Aulacorthum solani* on soybeans. Sublethal concentration (LC₅) of beta-cypermethrin accelerated the interspecific competition between the two species of aphids. Induced susceptibility of soybean plants to one species of aphids caused by the other species would be significantly impaired by the exposure to sublethal beta-cypermethrin.

Secondly, various pest species abundance and feeding strategies, i.e. the piercing-sucking aphid, the chewing caterpillar, the plant fungus and the root-knot nematode, were introduced on tomato plants, *Solanum lycopersicum* to evaluate the plant-mediated indirect interactions among these pests. In the results, insect performances, i.e. the number of aphid individuals and development rate of caterpillar larva, were correlated to the abundance of indirect interactions whether pests attacked tomato plants sequentially or simultaneously. When pests attacked tomato plants at the same time, insect performances were positively correlated to the abundance of indirect interactions. Nevertheless, when pests infested tomato plants in sequence, the insect performances were negatively correlated to the abundance of indirect interactions. However, such a correlation was not detected with the pathogenic organisms (fungi and nematodes).

Thirdly, the population dynamic of aphid *M. euphorbiae* was also monitored under a tri-trophic system. We found that the aphid population, involved in the above-belowground interactions, slowed down in both the short term and long term via reduced survival of nymph aphids and facilitating induction of winged aphids. Furthermore, parasitism efficiency of the natural enemy *Aphidius ervi* to aphids and tomato yields were also reduced by such above-belowground interactions.

Lastly, following an untargeted metabolomic approach, we detected many significant chemical variations between non-infested and infested plants, disregarding the pest infestation. Most of them are probably induced by the presence of nematodes, the only belowground pest. On the opposite, we did not observe many chemical differences in roots metabolome when we compared the different types of infestation, meaning that the presence of nematodes probably lead the metabolomic signal in infested roots, disregarding the presence of other pests aboveground.

All the studies may contribute to a comprehensive understanding of the diverse interactions among plants, pesticides, various pests, and natural enemies, and optimizing the integrated pest management in the agroecosystem.

Key words: trophic interaction; host-parasite interaction, plant-insect interactions; plant chemical defense; pest biodiversity; herbivorous insects; pathogenic fungi; nematodes

Acknowledgments

This PhD thesis was supported by a fellowship from the Chinese government and funded by the European Union's Horizon 2020 research and innovation program. I would like to thank all my supervisors who have contributed to the development of my project: Dr. Nicolas Desneux (INRA, Sophia-Antipolis) and Dr. Anne-Violette Lavoir (Université Côte d'Azur). This project would not have been a success without the collective efforts.

I first would like to express my gratitude to my supervisor, Dr. Nicolas Desneux, Research Director at INRA Sophia-Antipolis, who initiated and supervised this thesis, who has supervised me since 2013 when I was a Master student in China. He has encouraged and helped my work with enthusiasm. During these past years, he brought me the scientific support and made me benefit from his experiences. I would like also to thank Dr. Anne-Violette Lavoir who co-supervised me during my PhD study, both for the experiments and the writing of my thesis; I greatly benefited from her logic thinking in writing manuscripts.

I also thank Dr. Philippe Castagnone-Sereno for the support of nematodes and for his valuable advices on the working on tomato-nematode interactions. As well, I thank Dr. Philippe Claude Nicot (INRA, Avignon) who supports me with the colony of powdery mildew fungi for my experiment.

I thank Prof. Philippe Giordanengo (Université Amiens), Prof. Lucia Zappala (University of Catania), Dr. Anne-Violette Lavoir, Dr. Yvan Capowiez (INRA Avignon), Dr. Peng Han (Chinese Academy of Sciences) and Dr. Thomas Michel (Université Côte d'Azur) for agreeing to be the members of my PhD thesis jury. Especially, Philippe Giordanengo and Lucia Zappala who agreed to evaluate this work as Rapporteurs. I am especially grateful to them.

I thank all those who participated in the experiments. Many thanks to Philippe Bearez, Edwige Amiens-Desneux, Mateus Campos, Yusha Wang, Lionel Salvy, Roger Boll, Richard Brun for their technical support, encouragement and good humor. Also, big thanks to Dr. Fengluan Yao for his huge help to my thesis writing. I would thank Dr. Eva Thomine who provide warm care during my illness and give me great help to many document preparation for my Ph.D. defenses. I would thank Lucie Monticolli for the big help to the data analyses

for my experiments, and thank some other colleagues: Dr. Christine Becker and Ying Liu (Huazhong Agricultural Univeristiy) who have been actively involved in my experiments.

I also thank my former tutor, Dr. Dunlun Song, who helped and encouraged me to study abroad, and to Li Yang, Chen Luo, Xinyue Mei, Bin Wan, Yongpan Chen and all the other colleagues and friends for the very good ambience of the working environment.

Finally, I thank my parents and other family members who encouraged me to pursue this higher education abroad. I love all of you more than you can ever imagine.

Thanks to all! Sophia-Antipolis, France

Yanyan

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GENERAL INTRODUCTION

In the recent few decades of globalization, the threat of invasive species arriving at new places where they were previously absent is going to increase (Early et al., 2016; Levine and D'Antonio, 2003; Meyerson and Mooney, 2007; Perrings et al., 2005; Seebens et al., 2017). With increased globalization and connection by world trade, many new roads are open for invasive pests, such as the highly developed highway, rail transport network, the fast development of air transport and maritime transport, and the increased number of worldwide tourists (Hulme et al., 2009; Perrings et al., 2005). Both would result in an increase of pests in cropping system and farmers have to face more and more pests at the same time (Paini et al., 2016). Therefore, it's interesting and meaningful to work on the presence of many various pests in a single crop system.

Plants are hotbeds for a variety of pests which mainly include arthropod herbivores and phytopathogenic organisms such as viruses, bacteria, fungi and nematodes (Biere and Goverse, 2016; Stout et al., 2006). Most of the plant individuals are exposed to attack by various different herbivores and disease species during the period of seed germination, growth, development, bloom, and production of the plant (Karban and Baldwin, 1997). To protect their fitness from damage by these pests, plants have employed complex strategies, such as physical/chemical defenses, constitutive/induced defenses, and direct/indirect defenses (Kaplan et al., 2008a; War et al., 2018). Physical and chemical defenses are both vital strategies in plants to cope with biotic stress (Freeman and Brattie, 2008; Kariyat et al., 2013). The physical defense is the first barrier constructed by plants to protect from the attack by many pests (Hanley et al., 2007; War et al., 2012). Moreover, chemical defense, especially the chemically induced defense, is considered more effective to defend against biotic attackers (Mithöfer and Boland, 2012; War et al., 2012). Plants can produce an array of toxic proteins and metabolic substances which directly impact on the behavior, development, fecundity and survival of herbivores (Després et al., 2007; Kariyat et al. 2013; Sauge et al., 2010; Sharma et al., 2009; Zhao et al., 2009).

Moreover, Plant defenses can be either constitutive or induced upon attack by pests (Glynn et al., 2003; Kaplan et al., 2008a). Constitutive defenses are those that are prefabricated in plants and include many performed barriers, such as cell walls, waxy epidermal cuticle and bark (Freeman and Beattie, 2008; Glynn et al., 2003). Some of the constitutive defenses are operational at any time while others need an activation (Kant et al., 2015). Induced defenses are present only on attack by pests and are often subdivided into direct and indirect defenses (Chen, 2008). Direct defense includes the activation and production of some antifeedant

substances, such as toxins and proteinase inhibitors, which are detrimental to the growth, development or survival of plant attackers (Hartl et al., 2010; Howe and Jander, 2008). In addition, plants can also release chemical volatiles to attract the natural enemies of herbivores, such as parasitoids or predators (Kant et al., 2004; Schnee et al., 2006; Van Poecke et al., 2001).

After plants were attacked by pests, physiological traits of plants will be altered, which may have influences on other attackers sequentially or simultaneously arriving on the same plant (Gatehouse, 2002; Mouttet et al., 2011). Such plant-mediated indirect interactions largely depending on induced defenses may connect multiple pests that are both temporally and spatially separated on the host (Kaplan et al. 2008b; Poelman et al. 2008a, b; Soler et al. 2012a; Van Zandt and Agrawal 2004; Viswanathan et al. 2005). This interaction could be affected by numerous biotic and abiotic factors, such as attacking sequence, attacking location (shoot or root), pest density, feeding guilds of pests, plant species, temperature, drought, nutrient and even pesticides (Ali and Agrawal, 2014; Barber et al., 2015; Gaillard et al., 2018; Johnson et al., 2009, 2012; Kroes et al., 2016; Staley et al., 2007).

The plant-mediated indirect interactions have important consequence for both plants and their consumers to structure a complex food web in the community (Biere and Goverse, 2016; Stout et al., 2006; Thaler et al., 2004). Generally, with an increase of pest species presence on one plant, plant fitness would be at higher risk but the plant also evolved a compensation strategy to reduce the detrimental impact from pest attack (Järemo and Palmqvist, 2001; McNaughton, 1983). They can change assimilation rate, growth, resource allocation and morphs to compensate for the damage by multiple herbivore attackers (Barton, 2008; Robert et al., 2014; Tiffin, 2000). Moreover, the net effects of plant defenses on plant fitness result not only from the interaction between plants and their attackers, but also from the interaction web associated with the plant (Kant et al., 2015; Shiojiri et al., 2002).

The interactions among multiple plant attackers may be unidirectional or bidirectional and will lead to positive, negative or neutral impacts on plant challengers (Barber et al., 2015; Kroes et al., 2016; Johnson et al., 2009, 2012; Staley et al., 2007). For instance, herbivore chewing may deter the host from transporting carbohydrates away from the tissues of herbivore consumption (Delaney and Higley, 2006; Oppel et al., 2009). The strategy of resource conservation employed by herbivores may not only help them exploit a nutrient-rich part of the plant but also facilitate other species which compatibly colonize on the same tissue of host (Prokopy and Roitberg, 2001). In addition, one species would also benefit from another competing species that share the same host, via the suppression of defenses (Alba et al., 2015; Sarmiento et al., 2011). Furthermore, inhibition of plant defenses may interfere with the

attractiveness of natural enemies by decreasing the emission of the induced plant volatiles, thereby facilitating their competitors (Kant et al., 2008; Rodriguez-Saona et al., 2003; Sarmiento et al., 2011; Schwarzberg et al., 2011; Zhang et al., 2009). Not only may the indirect interactions have effects on individuals of plant consumers but they also would impact the pest population dynamics in both managed and natural ecosystem (Poelman et al., 2008; Stam et al., 2018; Stout et al., 2006).

The complexity would be added when the communities contains diverse pest species that can either induce or inhibit plant defenses (Alba et al., 2011). When plants are attacked by multiple pest species, besides the indirect interactions among pests, the interactions between herbivores and their natural enemies would be as well shaped by their integrated manipulation on plants (Alba et al., 2011; Kant et al., 2015; Van Dam et al., 2005). Volatile blends released by plants may be changed by multiple attack, and therefore lead to alteration in attractiveness to the natural enemies of insects (Battaglia et al., 2013). For instance, when cabbage plants are infested by both diamondback moth (*Plutella xylostella*) and cabbage butterfly (*Pieris rapae*), blend of volatiles emitted by the plant attacked by two pests is less attractive to diamondback moth's natural enemies (*Cotesia plutellae*) than that released by only one-caterpillar-infestation plant (Shiojiri et al., 2002). In turn, *P. xylostella* adult preferentially reproduce on cabbage plants which was previously infested by the cabbage butterfly, which may reduce the risk of parasitism in their progeny by natural enemies (Shiojiri et al., 2002). Not only the behavior, but also the survival, growth and development of the third trophic levels could be affected by plant defensive toxins that was taken in by their prey, i.e. herbivorous pests, through the trophic flow of food chains (Kant et al., 2015). Therefore, in addition to the interaction between plants and their pests, other organisms can as well benefit or suffer, either directly or indirectly, from defensive system regulated by plants (Ohgushi, 2005, 2008).

In this context, the aims of my Ph.D. study were to disentangle how the biodiversity affects plant-mediated indirect interactions among multiple pests involving different feeding strategies. In Chapter 1, an overview of the plant-mediated indirect interaction was described. Thereafter, in Chapter 2, all the biological models used in my Ph.D. study was introduced. In Chapter 3, we investigated impact of biotic (pest biodiversity, feeding guilds, attacking location, attacking sequence and host specialization) and abiotic factors (sublethal concentration of pesticides) on plant-mediated indirect interactions. Then, a long-term effect on the aphid population dynamics was monitored in a tri-trophic system (Chapter 4). Moreover, we would like to reveal the mechanisms underlying the plant-mediated indirect interaction and how such indirect interactions affect the aphid population dynamics and the attractiveness to parasitoids,

via the approach of metabolomics analyses (Chapter 5). In the last Chapter 6, general discussion and conclusion of the current study were performed and perspectives for the future study were proposed. All the studies would be useful to better understanding the plant-pesticide-pest-natural enemy interactions in the agroecosystem and optimize the biological control strategies for Integrated Pest Management programs.

CHAPTER 1. THE PLANT-MEDIATED INDIRECT INTERACTIONS AMONG PLANT BIOAGGRESSORS: A REVIEW OF MECHANISMS, MODULATING FACTORS AND IMPACT ON COMMUNITIES

Terrestrial plants play a key role in the sustainability of life on Earth to support almost all the living organisms for nutrients by fixing the solar energy. Plants can be consumed by plenty of parasites, including viruses, bacteria, fungi, nematodes, and herbivorous insects (Biere and Goverse, 2016). In this context, ‘pests’ indicate all the biotic aggressors including herbivores and phytopathogenic organisms. In the agroecosystem, plant-pest interactions are the most common interaction and also the main force driving the co-evolution between plant and their biotic consumers in nature (Howe and Jander 2008; Zhao et al. 2009). To protect themselves from the attack by pests, plants have developed an array of defensive strategies by diverse morphological and physiological modulations (Karban, 2011; War et al. 2012; Zhao et al. 2009). However, the biotic organisms have also evolved corresponding adaptive strategies to allow them colonized on their host successfully (Després et al. 2007; War and Sharma 2014). Therefore, the presence of one pest on the plant might have an indirect effect on another attacker through the physiological/ chemical alteration (nutritional quality or defense) in the host (Biere and Goverse, 2016; Stout et al., 2006). Understanding of the plant-pest associations would be in favor of the study on community ecology, especially on interactions among the members in the community (Stout et al., 2006).

1. Plant defenses against pests

1.1. Plant immunity to pathogens

To date, it has been known that two branches are involved in the plant immunity (Jones and Dangl, 2006). Transmembrane pattern recognition receptors (PRRs) were used in the first way to answer to pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs), such as flagellin, chitin, glycoproteins, and lipopolysaccharides, or endogenous plant-derived signals (Pieterse et al., 2012). In the second way, the polymorphic nucleotide binding-leucine rich repeat (NB-LRR) proteins encoded by the most *R* genes were employed inside the cell. The ongoing coevolution between plant immunity and microbial pathogens can be represented as a zig-zag models (Jones and Dangl, 2006; Fig. 1). Firstly, PAMPs or MAMPs are recognized by pattern-recognition receptors (PRRs). This results in the activation of PAMP-triggered immunity (PTI) that can stop further infestation by pathogens. Secondly, successful pathogens deployed effectors which interfered with the PTI and thereby

contributed to the pathogen virulence, leading to effector-triggered susceptibility (ETS). Thirdly, a given effector is specifically recognized either directly or indirectly by one of the NB-LRR proteins, causing effector-triggered immunity (ETI). In fact, ETI is an amplified PTI response, which contributes to an accelerated resistance to plant disease, e.g. resulting in a cell death (hypersensitive response) at the infection site. Fourthly, pathogen evolved new variations of effectors to evade recognition or inhibit ETI, thereby restoring the pathogen virulence and induced ETI again (Biere and Govere, 2016; Jones and Dangl, 2006). Recently, studies have been documented that the model applied as well to the interactions between plants and other organisms, such as beneficial microbes and plant-parasitic nematodes (Govere and Smart, 2014; Pieterse et al., 2012).

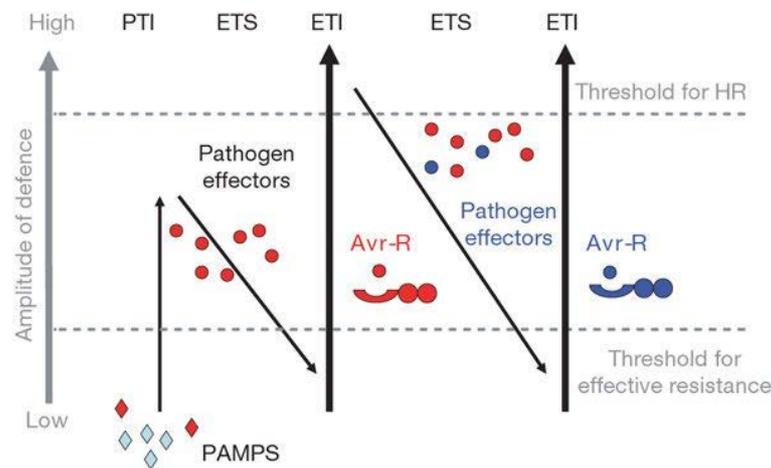


Figure 1. Illustration of the plant immunity system by a zig-zag model (from Jonathan and Dangl, 2006)

1.2. Plant defenses against herbivorous insects

During more than 350 million years coexisting with herbivores, a variety of defensive strategies, involving constitutive/induced defense, direct/indirect defense or physical/biochemical defense, have been developed by plants to withstand the consumption by insects (War et al., 2018; Fig. 2). The constitutive defense is always present in plants regardless of the external stimuli, while the induced defense is specifically elicited by certain aggressors (Sharma et al. 2009; War et al. 2012). In fact, both constitutive and induced defenses can be either direct or indirect (Mithöfer and Boland, 2012). Plants can directly defend against pests by their morphological features, such as thorns, prickles, high levels of lignification, increase in latex deposition or trichomes (Dalin and Björkman 2003; War et al. 2013a, b). Besides the function of trichomes serving as a mechanical barrier to prevent from insects, glandular trichomes can

secrete some compounds deterring herbivores from feeding or egg-deposition on the plant (Duke et al., 2000). Moreover, the biochemical-based defenses including various toxic secondary metabolites produced by the plant, was considered more effective due to the direct effects on herbivore growth and development (Karban, 2011; Kariyat et al., 2013; Kaur et al. 2015). Furthermore, they can also recruit natural enemies via herbivore-induced plant volatiles (HIPVs) and extrafloral nectar as indirect defensive strategy to cope with their biotic challengers (Arimura et al. 2009; Karban 2011; War et al. 2012).

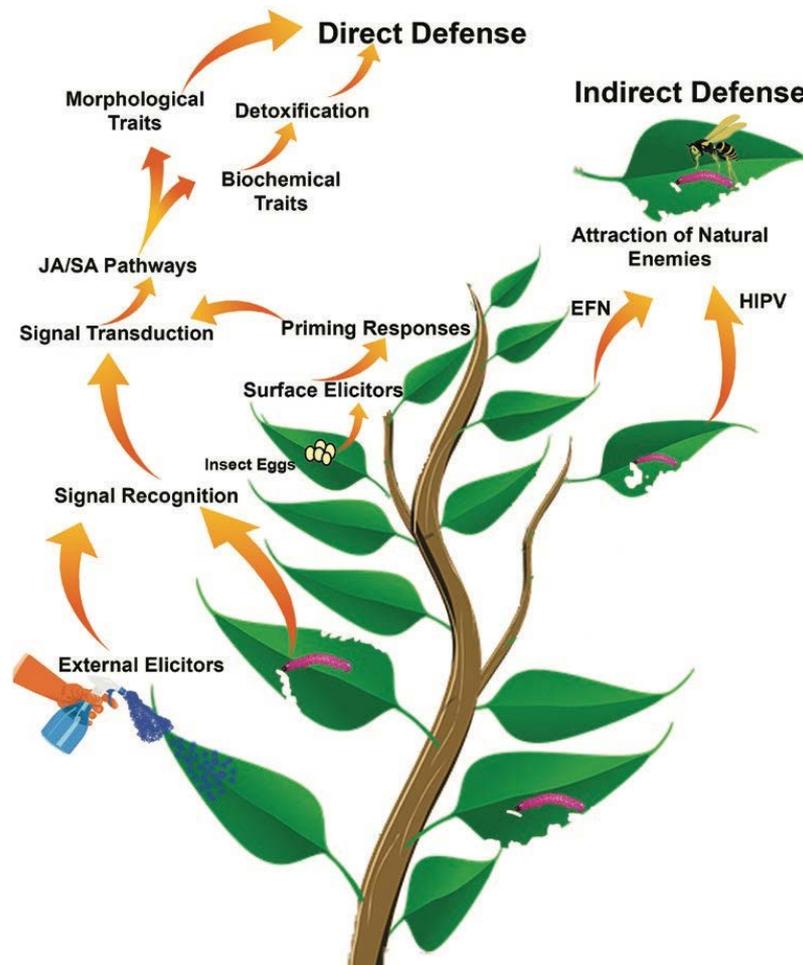


Figure 2. plant defense against herbivorous insects (EFN: extrafloral nectar; HIPV: herbivore-induced plant volatile; JA: jasmonic acid; SA: salicylic acid; from War et al., 2018)

1.3. Plant defense elicitors

Plants can recognize herbivore attack after the stimulation of the elicitors (Fig. 3), e.g. fatty acid-amino acid conjugates (FACs), in the oral secretion or oviposition fluid (Alborn et al. 2007; Howe and Jander, 2008; Schmelz et al. 2006). Volicitin (N-(17-hydroxylinolenoyl)-L-glutamine), was the first elicitor identified from the oral secretions of beet armyworm,

Spodoptera exigua. Volicitin caused the release of blends of volatiles to attract natural enemies of the caterpillar after application on the wounding maize (Alborn et al. 1997). N-linolenoyl-glutamine that belongs to the same family with volicitin, was isolated from the regurgitate of another lepidopteran larva, *Manduca sexta* (Wu et al., 2007). It had a potential to trigger mitogen-activated protein kinase (MAPK) pathway which is involved in plant development and could activate a series of defensive signaling pathways in the tobacco responding to herbivore attack (Wu et al., 2007). Inceptin and Caeliferins were also isolated from oral secretions of lepidopteran larvae and grasshopper species, respectively, and were both reported to be able to activate plant defensive pathways against herbivores (Alborn et al. 2007; Schmelz et al. 2006). Bruchins, produced by pea weevils, caused neoplastic growth at the site of weevil oviposition (Howe and Jander, 2008).

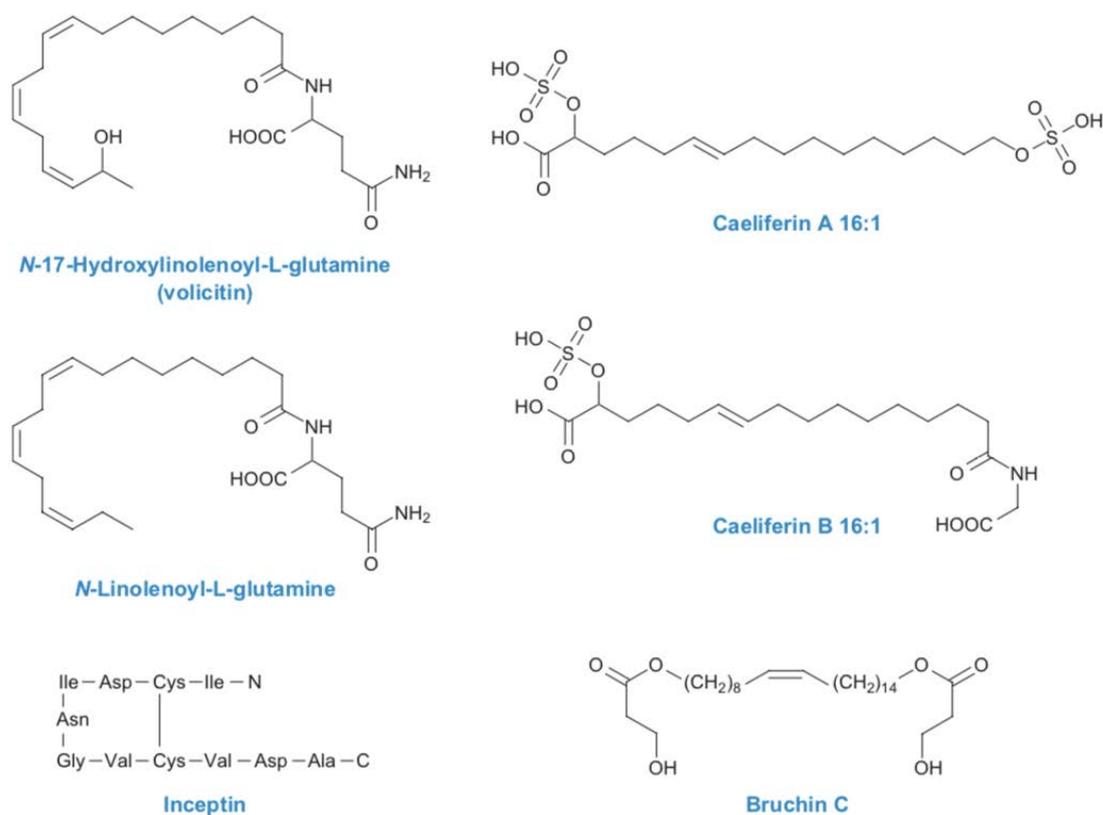


Figure 3. Insect-derived elicitors of plant defense responses (from Howe and Jander, 2008).

1.4. Hormone signaling and defense pathways

Plants recognize the plant defense elicitors from biotic stresses by activating kinase networks and defensive signaling phytohormones, mainly including jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) (Maffei et al. 2012; Pieterse et al. 2012). Signaling transduction pathways was induced by the pest attack, which results in the altered expression of defense-

related genes and, ultimately leads to the induction of biosynthesis pathways (Howe and Jander 2008; Maffei et al. 2012; Thaler et al. 2012). The induction of hormone-regulated defensive pathway largely depends on the feeding strategies of plant biotic challengers (Biere and Goverse, 2016). Generally, JA mediated the defensive signaling pathways against chewing insects and necrotrophic pathogens, and SA mediated the signaling pathways against phloem-feeding insects and biotrophic pathogens (Biere and Goverse, 2016). After attack by sap-feeding insects/biotrophic pathogens, SA would be synthesized and accumulated in the plant tissues, triggering translocation of the non-expressor protein of the pathogenesis-related genes 1 (*NPRI*) to the nucleus and finally leading to up-regulation of pathogenesis-related (*PR*) (Durrant and Dong, 2004). In JA-mediated signaling, jasmonoyl-isoleucine conjugate synthase 1 (*JAR1*) conjugates JA to the amino acid isoleucine (Ile) to form JA-Ile (Staswick and Tiryaki 2004). Biosynthesis of JA-Ile promotes the interaction between coronatin insensitive 1 (*COI1*) and jasmonate zim-domain (*JAZ*) proteins, which causes the proteolytic degradation of *JAZs* and de-repression of transcription factors, activating the expression of JA-mediated defense genes and leading to an induction of defensive secondary metabolites (Campos et al., 2014; Wasternack and Hause, 2013). JA signaling has two antagonistic branches, i.e. ethylene response factor (*ERF*) branch and myelocytomatosis protein (*MYC*) transcription factor branch, respectively (War et al., 2018). Hormone ET synergizes JA-*ERF* branch against chewing insects and abscisic acid (*ABA*) synergizes JA-*MYC2* branch against necrotrophic pathogens (Pieterse et al. 2012; War et al., 2018). In defensive system, SA- and JA-dependent signaling are interconnected and act antagonistically with each other, leading to hormone cross-talk (Pieterse et al. 2012). The SA/JA/ET core defensive system could be further antagonized with other growth and development phytohormones, including auxin (*AUX*), cytokinin (*CK*) and *ABA*. This may probably explain the trade-off in the plant between growth and defense (Biere and Goverse, 2016).

1.5. Defensive secondary metabolites

A variety of toxic substances, involving primary and secondary metabolites (Fig. 4), can be produced by plants upon pest attack (Mithöfer and Boland, 2012). Among these chemical compounds, secondary metabolites are staggering diverse and represent a major barrier to herbivore attack (Howe and Jander, 2008; War et al. 2012). In the recent few decades, more secondary metabolites have been suggested to have important ecological functions in plants: 1) protect plants against consumption by herbivores and against infection by microbial pathogens; 2) serve as attractants for pollinators and seed-dispersing animals, and as agents of plant-plant

competition, and as communicating signals with symbiotic microorganisms (Wink, 2018). Based on chemical structure, secondary metabolites can be classified into three main groups: phenolics (with the common feature of phenol rings and including phenolic acids, coumarins, flavonoids, tannins and lignin), terpenoids (mainly composed of plant volatiles, cardiac glycosides, carotenoids and sterols), and nitrogen-containing compounds (extremely diverse, mainly consisting of alkaloids, glucosinolates, cyanogenic glycosides, and nonprotein amino acids) (Chowański et al., 2016).

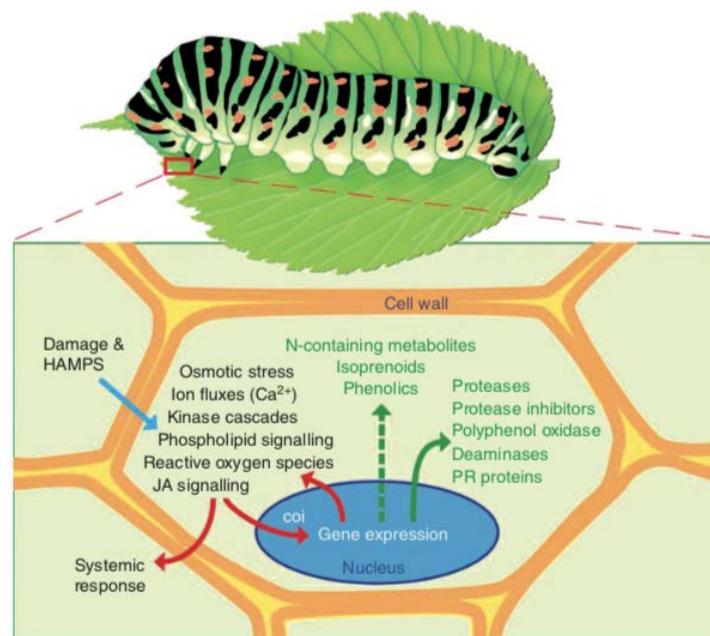


Figure 4. The plant defense responses. Wounding or exposure to oral secretions from caterpillar larvae leading to osmotic stress, causes ion fluxes and elicit signaling cascades, thereby leading to rapid accumulation of jasmonates (JAs). JAs enters the nucleus, where it associates with a protein complex including its receptor encoded by the coronatine insensitive (*coi*) gene. Binding to this complex starts degradation of transcriptional suppressor proteins, thereby activating defense gene expression. JAs as well stimulates increased accumulation of secondary metabolites which are toxic and insecticidal to herbivores and result in plant tissues less palatable. Upstream processes are shown in black and the defensive compounds produced by the plant are in green (from Kant et al., 2015).

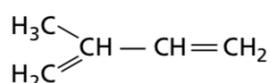
1.5.1. Phenolic compounds

Single phenolics consist of an aromatic ring with one or more hydroxyl groups and they can be polymerized to form polyphenols (Zhao et al., 2009). Both can be additionally modified, which

results in to a vast quantity (>9000) of chemically diverse metabolites, including benzoquinones, phenolic acids (e.g. SA), coumarins, flavonoids, lignins and tannins (Balasundram et al., 2006). Phenolics are constitutively produced in plant probably linked to their various functions in primary metabolism. For example, flavonoids are crucial for plant reproduction because they are necessary for pollen development and provide visual cues to attract pollinators and seed dispersers (Hoballah et al., 2007; Jaakola et al., 2002; Van der Meer et al., 1992). Although they are constitutively present, their concentration is induced in response to herbivore attack as reported in many plants, such as coffee, castor, cotton, tomato and black gram (Bhonwong et al. 2009; Dixit et al. 2017; Magalhães et al. 2008; Rani and Ravibabu, 2011; Taggar et al. 2014). Phenolics prevent plant from herbivore attack due to their direct toxicity to insects and/ or the feeding-deterrent nature (Dixit et al. 2017; War et al. 2013b). For instance, cucurbitacins are bitter in taste and they make plants unpalatable and hostile to a large scale of herbivores, including lepidopteran larvae, beetles and mites (Agrawal et al. 1999; Balkema-Boomstra et al., 2003; Tallamy et al. 1997). Not only the insect growth and development can be affected, but fertility of herbivores is also impacted by deterring oviposition (Agrawal et al. 1999; Balkema-Boomstra et al. 2003; Tallamy et al. 1997). Furthermore, phenolic compounds are induced to accumulation and activity of polyphenol oxidase and peroxidase increase in plants upon attack (Constabel et al., 2000; Stout et al., 1999). Such amplified chemical defense responses coincide with the deposition of lignin at the site of infection or attack, resulting in an extra physical barrier, which is especially effective against small organisms such as nematodes and some immobile arthropods (Valette et al., 1998). Additionally, some volatile phenolic compounds, such as methyl salicylate, are involved in the indirect plant defense to attract natural enemies of herbivorous insects (Ament et al., 2004, 2010; Heil, 2008).

1.5.2. Terpenoids

Terpenoids comprise of the largest class of secondary products and are derived from the basic structural element of ‘isoprene units’:



Terpenes are classified by the number of five-carbon units they contain. Ten-carbon terpenes, which include two C₅ units, are called monoterpenes; 15-carbon terpenes (with three C₅ units) are sesquiterpenes; and 20-carbon terpenes (with four C₅ units) are diterpenes. Larger terpenes

include triterpenes (30 carbons/ six C₅ units), tetraterpenes (40 carbons/ eight C₅ units), and polyterpenoids ([C₅]_n carbons, n > 8) (Gershenzon and Engelberth, 2010).

The major players in terpenoid volatiles are represented by monoterpenoids, sesquiterpenoids, and homoterpenoids, which all significantly contribute to the blend of plant-derived volatiles (Mithöfer and Boland, 2012). Due to the highly lipophilicity of volatile terpenoids, they can penetrate plasma membranes and increase their permeability, causing direct toxic and repellent effects on arthropods (Bleeker et al., 2012; Sikkema et al., 1995, Vaughan et al., 2013). Essential oils, the mixtures of volatile monoterpenes and sesquiterpenes found in many plants, are well-known to repel insects (Gershenzon and Engelberth, 2010). Menthol and limonene, which are the chief monoterpene constituent of peppermint oil and lemon oil respectively, have been reported to be able to defend against insects (Regnault-Roger et al., 2012). Moreover, upon attack by herbivores, plants can also emit volatile terpenoids to attract natural enemies of insects as an indirect defensive strategy (Kant et al., 2009). In addition to the interactions with herbivores and their natural enemies, terpenoids can also interfere with neighboring plants. For example, carvacrol, a monoterpene substance, serves as an allelochemical to inhibit respiration, growth and seed germination and to block the nitrogen cycle of neighboring plants (Maffei et al., 2012). Among the nonvolatile terpene antiherbivore compounds, *azadirachtin*, a limonoid of triterpenes (C₃₀), are well known as bitter substances/ powerful deterrents to prevent from insect consumption (Gershenzon and Engelberth, 2010). Although considered highly effective, terpenoid-mediated indirect defense probably backfire because of the potential risk to attract even more herbivores (Kessler and Baldwin, 2001; Runyon et al., 2006).

1.5.3. N-containing compounds

A large variety of plant secondary metabolites with nitrogen in their structure are categorized into N-containing compounds, mainly including alkaloids, cyanogenic glycosides, glucosinolates, and nonprotein amino acids (Gershenzon and Engelberth, 2010). Alkaloids which contain at least one nitrogen atom in a heterocyclic ring constitute the biggest subgroup (>12 000 compounds) (Levin, 1976). Some amino acids, purine nucleotides and isoprenoids are the precursors of alkaloids (Itkin et al., 2013). Plants from *Solanaceae*, *Papaveraceae*, *Apocynaceae*, and *Ranunculaceae* families are often rich in alkaloids (Mithöfer and Boland, 2012). Glycoalkaloids, the specific secondary metabolites in *Solanaceae* plants, have been wide reported to play a vital role in plant defense against herbivorous insects and pathogenic organisms (Friedman, 2002; Gelder, 1991; Korpan et al., 2004; Lachman et al., 2001; Laurila

2004). Although alkaloids are also constitutively present in plants, attack by herbivores can increase their production and transport to the other parts for plant defenses (Baldwin, 1988). They can act on many organisms by affecting enzymes, altering different physiological processes; intercalating with nucleic acids, inhibiting DNA synthesis and repair; or strongly affecting the nervous systems (Mithöfer and Boland, 2012). Moreover, cyanogenic glucosides are present in many plants to protect them against herbivores, via emission of volatile hydrogen cyanide (HCN), causing inhibition of cellular respiration (Way, 1984). Glucosinolates as well as cyanogenic glucosides, are derived from amino acids and are mainly found in *Brassicaceae* plants (Halkier and Gershenzon, 2006; Hansen et al., 2001). Attack by herbivores triggered the production and release of isothiocyanates and nitriles (reactive hydrolysis products of glucosinolates), which may be directly toxic and repellent to herbivores, serve as attractants of parasitoids and probably also attract specialist herbivores (Beran et al., 2011; Bones and Rossiter, 1996; Lazzeri et al., 2004; Mumm et al., 2008).

1.6. Pests respond to plant defenses

Along with the development of signaling networks regulating induced defenses and the diversity of secondary metabolites, pests have as well evolved a variety of strategies to cope with plant defenses, including adaption to defenses (Fig. 5) and suppression of defenses (Alba et al., 2011). Firstly, Herbivores can avoid the defended plant tissues as much as possible or remove some defensive structures, e.g. trichomes and latex channels (Cardoso, 2008; Paschold et al., 2007; Perkins et al., 2013; Rodrigues et al., 2010; Shroff et al., 2008). Secondly, defensive compounds can be metabolized by a variety of detoxifying enzymes in herbivores to decrease the exposure to toxins. A wide range of allelochemicals, involving in furanocoumarins, flavonoids, terpenoids, alkaloids and glucosinolates, can be metabolized by Cytochrome P450s of herbivorous insects (Després et al., 2007; Feyereisen, 2012). In addition, it was reported that Glutathione S-transferases (GSTs) are able to metabolize a variety of thiocyanate conjugates in the larvae of some generalist insect, such as *Spodoptera frugiperda*, *Trichoplusia ni*, *Anticarsia gemmatalis* (Kant et al., 2015). The diversity of glucosinolate-derived thiocyanates that insects are able to metabolize could be correlated with their host plant range (Li et al., 2007). Furthermore, many herbivores can use plant defensive substances for their own defense against the natural enemies by storing the ingested chemicals in integument or other specialized tissues, which may interfere with the indirect defense of plants (Kant et al., 2015; Nishida, 2002; Opitz and Müller, 2009). Also, some insect species have evolved additional means to further utilize the sequestered compounds for their own defenses. For example, flea beetles

Phyllotreta striolata evolved a specific myrosinase to convert the toxic glucosinolates into nontoxic products and release these as toxic and repellent volatiles into the air (Beran et al., 2014; Rahfeld et al., 2014). Thirdly, a variety of pests have evolved the means to suppress plant defenses by interfering with the ongoing physiological process of induced plant defenses (Musser et al., 2002). Defenses can be suppressed by modulating the upstream or downstream of a defensive pathway, blocking it altogether or inhibiting it to intermediate levels, which finally reduces the rate of production of defensive substances (Alba et al., 2015). Suppression of plant defenses is a well-known phenomenon in plant pathogens, including pathogenic bacteria, fungi, oomycetes and nematodes (Abramovitch et al., 2006; Kamoun, 2006; Voegelé and Mendgen, 2003). For instance, the root knot nematode *Meloidogyne incognita* was reported to suppress SA- and JA-dependent systemic acquired resistance in *Arabidopsis thaliana* (Hamamouch et al., 2011). Additionally, many herbivores, such as *S. exigua*, *Pieris brassicae* and *Macrostelus quadrilineatus* are recently documented to be able to suppress JA-mediated and/or SA-mediated defensive pathways (Consales et al., 2012; Sugio et al., 2011; Weech et al., 2008).

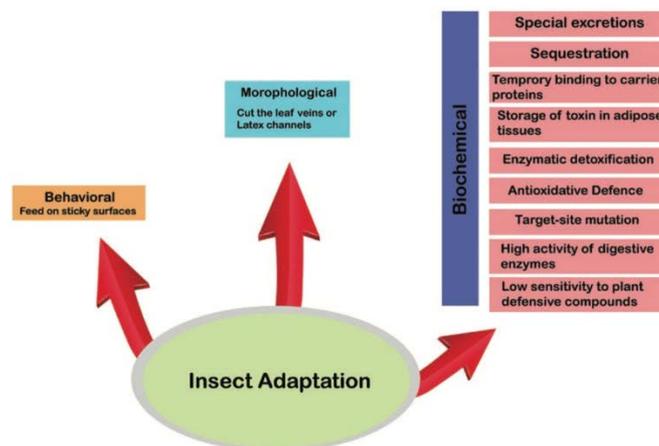


Figure 5. Adaptions of herbivorous insects to plant defenses (from War and Sharma, 2014).

2. Mechanisms underlying plant-mediated indirect interactions among herbivores

Elucidating the mechanisms underlying plant-mediated indirect interactions is vital to comprehensively understand and predict the outcomes of such interactions among multiple plant attackers. However, the mechanisms are diverse, probably involving plant defenses (induced/suppressed), interactions between hormone pathways, alterations in primary

metabolism, source-sink relationships, resource allocation and morphology (Biere and Goverse, 2016).

2.1. Mechanisms based on plant defenses

Responses induced by one pest species may cause resistance/susceptibility of the host plant to another species due to the induced/suppressed plant defenses (Moultet et al., 2013; Poelman et al., 2008). However, plant-induced defenses may be a major determinant of such indirect interactions among different plant consumers. Both local and systemic induced defenses could be activated by the first bioaggressor attack, leading to the production of various toxic compounds, e.g. secondary metabolites, which would negatively affect the subsequent attackers on the same host (Stout et al., 2006). For instance, development of *Pieris rapae* larvae significantly reduced due to higher foliar concentration of glucosinolate, when *Brassica nigra* plant roots were also infected by the nematode *Pratylenchus penetrans* (Van Dam et al., 2005).

The distinct defensive signaling pathways that largely depend on the pest feeding strategies may interact directly and indirectly, forming complex networks, and these interactions could be probably additive, antagonistic or synergistic (Koornneef and Pieterse, 2008). Interactions between the hormone pathways triggered by the plant consumers would shape outcomes of the plant-mediated indirect interactions among the pests which share the same host (Stout et al., 2006). JA and SA as the most important hormone signalings that mediate plant induced defenses against the biotic challengers of plants, are commonly antagonized with each other (Caarls et al., 2015; Robert-Seilaniantz et al., 2011; Thaler et al., 2012). Therefore, owing to the cross talk between JA and SA pathways, bioaggressors are predicted to be negatively impacted by other attackers with the same feeding guild, while benefit from those with a different feeding strategy. For example, it was documented that infestation by pathogens *Pseudomonas syringae* on tomatoes induced both JA, SA pathway and proteinase inhibitors (PIs), causing reduced growth of the larvae of caterpillars *S. exigua* which only trigger JA-mediated signaling pathway. Conversely, infection by tobacco mosaic virus (TMV) triggered SA pathway alone in tomatoes, which facilitated *S. exigua* growth but suppressed the population growth of aphid which as well activated SA-mediated defensive pathway (Thaler et al., 2010). However, the antagonism between JA and SA is not unconditional. Leon-Reyes et al. reported that only when SA pathway is activated earlier or simultaneously than the JA pathway, can SA inhibit the JA signaling in *Arabidopsis* (Leon-Reyes et al., 2010a, b). In addition to the activated sequence of hormones, the interplay between signaling pathways still depends on the activated location when plants are attacked by multiple

pests (Caarls et al., 2015; Leon-Reyes et al., 2010a; Thaler et al., 2012). This indicates that the simultaneous induction of various distinct signaling pathways may affect community members differently.

2.2. Alteration of resource dynamics in plants

In spite of the recent emphasis on plant-induced defense, other changes in plant nutritional resources may be as important as the induced secondary metabolism in mediating the indirect interaction among multiple bioaggressors. Attack by fungi, herbivorous insects, and nematodes not only results in the induction of defensive responses in plants but also causes strong alterations in primary metabolism, which can profoundly affect other organisms present the same host (Berger et al., 2007; Zhou et al., 2015). Numerous studies have reported that infestation by herbivores or pathogens changed the level of free amino acid and carbohydrates of plants, which had an influence on the performance of other insects sharing the same host (Hare and Dodds, 1987; Johnson et al., 2003). For instance, infection by nematodes in tomato roots reduced the concentration of nitrogen in foliage, leading to suppression on whitefly population on tomato leaves (Guo and Ge, 2017).

Pathogens and sucking-piercing insects attacking plants could turn source tissues which produce assimilates into sink tissues (Lemoine et al., 2013; Stout et al., 2006). This protects resources for the local attackers but results in a negative effect on carbohydrate supply to organisms in systemic tissues. Moreover, chewing herbivores feeding on foliage generally caused increased photosynthates allotting to roots (Erb et al., 2009; Orians et al., 2011). This ‘herbivore-induced resource reallocation’ strategy could transport assimilates from the damaged tissues to ‘safe tissues’ which are inaccessible to herbivore consumption and restore the photosynthates for regrowth after herbivore attack stop (Biere and Goverse, 2016). However, storage is not the only destination of diverted resources which may be also used for defense or growth of the plant. Hence, resource reallocation caused by foliar herbivores may either facilitate or deter the infestation of organisms in roots. For example, leaf-chewer *Ostrinia nubilalis* on the leaves of corn reduced the penetration of nematode *M. incognita* into roots (Tiwari et al., 2009). However, *Heterodera glycines* benefited from the defoliation of *Helicoverpa zea* on soybean leaves (Alston et al., 1993).

3. Modulating factors of plant-mediated interactions

The outcomes of plant-mediated interaction may depend on numerous biotic and abiotic factors that can affect the organism members involved in such interactions.

3.1. Biotic factors

Plant-pest interaction largely rely on the feeding mode, host specialization, attacking sequence and attacking location of bioaggressors, which ultimately shape the outcome of the interactions among pests present in the same host.

3.1.1. Feeding strategy of pests

Phytochemical defense responses in plants triggered by different attackers are often affected by the feeding guilds of plant challengers. As discribed in *Section 1.1.4*, defoliation by chewing herbivores caused an induction of defensive secondary metabolites generally regulated by JA pathway (Fonseca et al., 2009; Howe and Jander, 2008). However, sap-feeding insects on the phloem, often activate SA pathway which can interfere with JA pathway (Gao et al., 2007; Pieterse et al., 2012). Herbivores of the cell-content feeding species such as spider mites, may likely trigger both the SA- and JA- signaling pathways (Ament et al., 2004; Kant et al., 2004; Leitner et al., 2005; Ozawa et al., 2000). In contrast to herbivorous insects, biotrophic plant pathogens generally induce SA-regulated signaling pathway, leading to the activation of systemic acquired resistance (SAR) and production of parhogenesis-related (PR) proteins (Clarke et al., 2000; Di et al., 2017; Nawrath and Mettraux, 1999) while response to necrotrophic pathogens depends more on JA-mediated defense (Biere and Goverse, 2016; Glazebrook, 2005; Thomma et al., 1998). Nevertheless, knowledge on hormone-regulated defenses triggered by nematodes is more complex and still fragmentary. As reported, JA- and/or SA-mediated signaling pathway could be induced after root-knot nematode invasion in plant roots (Bhattarai et al., 2008; Guo and Ge, 2017; Nahar et al., 2011; Zhao et al., 2018).

Therefore, plant-mediated interaction among multiple pest species with different feeding modes could cause interplay between distinct signaling pathways, thereby leading to the effect on the outcome of such indirect interactions. Despite the fact that the feeding strategies of bioaggressor allow some predictions on defensive response in plants, study results are often inconsistent. For instance, some caterpillars and beetles were found to compromise the induction of JA- or SA-mediated defenses, while some nematode was reported to activate both JA and SA defensive pathways (Alba et al., 2015; Chung et al., 2013; Guo and Ge., 2017; Musser et al., 2002; Sarmiento et al., 2011).

3.1.2. Host specialization of pests (generalist/ specialist pests)

So far, the view on whether induced defenses are consistently dependent on the degree of herbivore specialization is still debated, thereby leading to the inconsistent effects of host specialization on plant-mediated indirect interactions (Agrawal, 2000; Hopkins et al., 2009; Mewis et al., 2006; Poelman et al., 2008b; Ratzka et al., 2002; Reymond et al., 2004). Generalist pests are the ones that can feed on a wide range of plant taxa while the specialists are able to feed just a few or even a single-plant species, and the specialists are frequently less impacted by plant defenses than the generalist (Agrawal and Kurashige, 2003; Travers-Martin and Mueller, 2007). However, there has been a long-standing hypothesis that specialist and generalist pests interact with plants in different ways, including plant defenses induced by them (Dussourd and Denno, 1994; Poelman et al., 2008b). The distinct induction of plant defenses triggered by specialists and generalists would contribute to their interactions on the same host. To verify this hypothesis, many studies were conducted at the transcriptional metabolic, hormone signaling pathway/phytochemical, and ecological levels, respectively (Agrawal, 2000; Diezel et al., 2009; Figon, 2013). Firstly, in transcriptional responses to three herbivores within the same feeding guilds (leaf chewer from *Lepidoptera*), plants responded more similarly to infestation by the two generalists (*S. exigua* and *Heliothis virescens*) than to infestation by the specialist (*M. sexta*). Also, the distinction in transcription between specialist and generalist coincides with the difference in FACs (elicitors of induced defense) composition from the three species of herbivores (Voelckel and Baldwin, 2004). Secondly, Figon also found that the specialist caterpillar *M. sexta* activated JA and ET signaling pathways in *Nicotiana attenuate*, while SA pathway was triggered by the generalist *S. exigua* attacking on the plant (Figon, 2013). Thirdly, within the same guild of feeders (leaf-chewing caterpillar), some herbivores can respectively cause induced resistance, no influence, or induced susceptibility to the subsequently feeding caterpillars. The plant-mediated interactions were asymmetric between the generalist *Trichoplusia ni* and the specialist *P. xylostella* on the wild radish. Also, attack by either the specialist *P. xylostella* or the generalist *S. exigua* conferred a resistance on the wild radish plant to the specialist *Pieris rapae*, while plant damage by generalist *T. ni* did not affect the performance of the specialist *P. rapae* (Agrawal, 2000).

Although generalist and specialist herbivores may induce differential responses in plants and result in distinct effects on the plant-mediated interactions, impact caused by the same degree of diet specialization of insects could be also inconsistent. In the same study of Agrawal (2000), infestation by the generalist *S. exigua* caused a resistance of the host to other two specialist *P. rapae* and *P. xylostella*, whereas the other generalist *T. ni* did not induce a resistance to these two specialist species (Agrawal, 2000). Thus, the extent to which the

differences are driven by the host specialization of the herbivores could not be easily decided, because only one or two species for each (specialist/generalist) are involved in most of the studies (De Vos et al., 2005; Govind et al., 2010; Poelman et al., 2008b; Reymond et al., 2004; Travers-Martin and Mueller, 2007; Voelckel and Baldwin, 2004). More species should be involved to make the paradigm more applicable. Moreover, although some of studies provided evidence of distinction in plant defenses induced by specialist and generalist herbivores, other study results suggest that these differences may be not solely due to the diet specialization of herbivores (Govind et al., 2010; Lankau, 2007; Mewis, et al. 2006; Poelman et al., 2008b; Reymond et al., 2004).

3.1.3. Spatio-temporal factors

Induced plant defensive compounds accumulate not only at the local feeding site, but also in undamaged tissues, and these ‘systemic’ responses cause that plant-mediated interactions are spatiotemporal-dependent. The order of herbivore arrival on the plant can greatly impact the herbivore performance and even diversity of herbivore species occurring in the later season (Erb et al., 2011; Van Zandt and Agrawal, 2004; Viswanathan et al., 2005). For example, in maize *Zea mays*, the leaf-chewer insect *S. fugiperda*, had a significant negative effect on the colonization of the root feeder *Diabrotica virgifera* when they first attacked the plant, but the aboveground herbivore did not impact the performance of the root attacker when *S. fugiperda* arrived later than *D. virgifera* (Erb et al., 2011). When aphids *B. brassicae* and caterpillars *Pieris brassicae* attacked *Brassica oleracea* together, performance of caterpillars benefited more from the simultaneous attack than sequential attack by the two species of herbivores (Soler et al., 2012b). The sequence of herbivore attack is probably linked to different expression of JA- and SA-related genes and the transcriptional changes, leading to distinct outcome of interplay between different signaling pathways (Biere and Goverse, 2016; Soler et al., 2012a, b).

Not just the order of attack resulted in different plant responses, affecting plant-mediated interactions, but different locations attacked by pests can also affect the interactions, due to distinction in induced defenses between shoot and roots (Balmer and Mauch-Mani, 2013). For instance, root feeding by wireworms *Agriotes lineatus* largely reduced leaf consumption by the aboveground chewing insect *S. exigua*, via increasing the terpenoid defensive substances in cotton leaves (Bezemer et al., 2003). Aboveground herbivore *S. exigua* only induced defensive terpenoids in young leaves, while attack by root herbivores induced terpenoids in all leaves, indicating the advantage of root-induced leaf defense compared to shoot-induced leaf defense

(Bezemer et al., 2003). Thus, the attacking location where the interaction is initiated (root or shoot) plays an important role in the outcome of the indirect interaction, which can result in the asymmetry interactions between aboveground and belowground pests, depending on the locations of the inducer and responder (Biere and Govere, 2016).

3.2. Abiotic factors

In addition to the biotic factors, interactions between plants and herbivorous insects or plant pathogens can be strongly influenced by numerous abiotic stresses, such as draught, heat, cold, nutrient limitation and salt (Abouelsaad, 2016; Ballhorn et al., 2011; Johnson et al., 2009; Staley et al., 2007; Wani et al., 2016). Especially, water and nitrogen as the most important nutritional resources tightly linked to the plant physiology have strong effects on the plant inducible defenses against phytophagous pests (Staley et al., 2007).

3.2.1. Water

Water stress including drought and flooding play a key role in impacting the outcome of both plant-insect and plant-pathogen interactions (Rosenzweig et al., 2001). Plants response to the water stress, especially draught, mainly regulated by the stress hormone, abscisic acid (ABA), which can activate the transcriptional factors and downstream functional genes to rebuilt homeostasis in the plant (Asselbergh et al., 2008; Bostock et al., 2014; Harb et al., 2010; Ramanjulu and Bartels, 2002). Besides the important role in response to abiotic stress, ABA also has effects on plant resistance to herbivores and pathogens through antagonistically or synergistically interacting with phytohormonal pathways of plant defenses (Asselbergh et al., 2008; Mohr and Cahill, 2007; Thaler and Bostock, 2004).

Moreover, draught could also change quality of nutrient in the phloem of plants (Huberty and Denno, 2004). It has been documented that performance of herbivores, e.g. aphids, can be positively, neutrally or negatively affected by drought (Khan et al., 2010; McVean and Dixon, 2001; Pons and Tatchell, 1995). Additionally, the root herbivore may remove fine roots which are responsible for water and nutrient uptakes, leading to a relative reduction of water content and an increase in soluble nitrogen and carbohydrate of the foliage (Masters et al., 1993). This result could subsequently facilitate the performance of aphids, a sap feeder on plant phloem. Soil water availability, therefore, may have the capacity to affect the strength and occurrence of these interactions between aboveground and belowground herbivores (Staley et al., 2007). For instance, drought enhanced the interaction between a root feeder, the larva of *Phyllopertha horticola* and the aphid *Aphis fabae*, by enhancing the positive effects of root herbivores on

aphid fecundity and developments. In contrast, the reduced interaction between herbivores could also result from draught. The root-chewing wireworm larvae reduced the performance and abundance of the leaf miner *Stephensia brunnichella* on the same host plant *Clinopodium vulgare*, but such negative effects of belowground herbivores on aboveground insects did not occur when the host was exposed to a severe drought (Staley et al., 2007). The opposing effects of water stress on plant-mediated interactions may be due to distinctions in the mechanisms how the herbivores interact with the host.

3.2.2. Nitrogen

Among all the nutritional resources requested by plants, nitrogen play a prominent role in all the plant biological process, including photosynthesis, growth and development (Alvarez et al. 2012; Porter and Lawlor 2009; Ramage and Williams 2002). Studies were reported that nitrogen limitation/fertilization may cause the alteration of concentrations of numerous plant metabolites, resulting from the changed expression of a sequence of genes related to plant primary and secondary metabolism (Gerendás et al., 2008; Mutikainen et al., 2000; Vidal and Gutiérrez, 2008; Xu et al., 2010). Such physiological changes of plants can have an influence on the behavior, growth, development, survival and population of herbivorous insects, via the altered constitutive and inducible chemical defenses and modified plant biomass (Glynn et al., 2003; Sauge et al., 2010; Staley et al., 2009, 2011). Furthermore, nitrogen availability may affect the plant-mediated indirect interactions between herbivores, either infesting the same plant compartment or different plant compartments. For example, the leaf-chewer *Plutella xylostella* reduced population of the sap-feeder aphid *Brevicoryne brassicae* on *B. oleracea*, but the competed suppression on aphid fecundity did not occur when the host was supplied with ammonium nitrate fertilization (Staley et al., 2011). In addition, the increase of nitrate supply to *B. brassicae* altered the positive effects of aphids on nematode infection to the negative (Kutyniok and Muller, 2013).

4. Impact on associated communities

Plant-mediated interactions among multiple pest species may have crucial effects on community organization within an agroecosystem. In fact, any plant attacker that can induce plant defense may have a potential effect on all other community members and ultimately alter plant-associated community over the entire season (Bukovinsky et al., 2010; Poelman et al., 2010).

4.1. Impact on pests

Pests present in the same host could be linked by the induced plant responses, thereafter leading to effects on other attackers on the host. The plant-mediated effects may be asymmetric on performance of pests involved in the indirect interactions (Poelman and Dicke, 2014). When monarch caterpillars *Danaus plexippus* and oleander aphids *Aphis nerii* co-attacked the milkweeds, aphid population was significantly suppressed by caterpillars, but the caterpillar development benefited from the co-infestation (Ali and Agrawal, 2014). Biochemical changes induced by the plant attackers can subsequently impact the attraction, feeding behavior, development and fecundity of any other pests present on the same host (Biere and Goverse, 2016). Plant pre-infested by the green peach aphid, *Myzus persicae* significantly increase the attractiveness of potato plants to the potato aphid, *Macrosiphum euphorbiae* (Brunissen et al., 2009). Moreover, continuous infestation by *M. persicae* individuals on potato plant significantly increased nymph survival of *M. euphorbiae* and the host pre-infested by *M. persicae* remarkably shortened the development of *M. euphorbiae* (Brunissen et al., 2009). Furthermore, plant defenses induced by the early-season insects resulted in behavioral or physiological modulations in herbivore individuals, thereby may significantly impact their population dynamics in long term (Poelman et al., 2008).

4.2. Impact on plants

The consequence of multiple biotic attackers on plants may be complex and the interactions between plant consumers could result in synergistic or antagonistic effects on the plant fitness (Biere and Goverse, 2016; Stout et al., 2006; Thaler et al., 2012). Plants have to operate different defensive responses against their challengers, which may be costlier in terms of resources than a single defense, thereby affecting plant performance (Hauser et al., 2013). Upon attack, the damaged plant emitted some organic volatiles that may reach neighboring plants and induce responses in the non-infested plants before herbivores arrive, thereby preventing the neighboring ones from herbivore attack (Baldwin et al., 2006). Colonization by a less ravaging tobacco hornworm conferred tobacco plants more resistance to mirid bug, leading to reduction of plant fitness loss caused by the latter bug attack (Kessler and Baldwin, 2004). Thus, the plant fitness and its responses to early-season herbivores should be assessed in the context of community-wide consequences of the plastic plant phenotype. To date, study on the evaluation of plant-mediated species interactions on the plant fitness is still limited (Utsumi, 2011).

4.3. Impact on natural enemies

Carnivorous natural enemies of herbivores play an important role in insect communities by reducing populations of herbivorous insects, therefore benefiting plants (Poolman and Dicke, 2014). During the predation/parasitism of predator/ parasitoids, plants can make an indication for natural enemies to quickly hunt for their prey/host by the release of herbivore-induced plant volatiles (Hare, 2011; Kessler and Heil, 2011; Mumm and Dicke, 2010). Therefore, interactions with the third trophic level may be as well affected when multiple herbivore are present in the same host, via alteration of blends of plant volatiles and trophic cascades (Kant et al., 2015). For instance, when cabbage plants are simultaneously infested by multiple herbivorous organisms, the new blend of volatiles emitted by the plant is less attractive to the natural enemies than that released by the singly infested plant (Shiojiri et al., 2002). Moreover, another study reported that the development of *P. brassicae* larvae was slower on wild mustard *Brassica nigra* plants that were jointly infested with the cabbage root fly *Delia radicum*, and thereafter reduced developmental rates of the natural enemy *Cotesia glomerata* (Van Dam et al., 2005). In contrary, Johnson et al found that the root-feeding weevils *Otiorhynchus sulcatus* increased the abundance of aphid natural enemies, following the 7-fold increase in aphid population on blackcurrants (Johnson et al., 2013).

Overall, plants are confronted with various biotic and abiotic stresses which would impact the interactions between plants and their consumers, causing the further impact on the plant-mediated indirect interactions among the bioaggressors. To date, the major biotic factors studied on such indirect interactions are pest feeding strategies, host specialization, attacking location and attacking sequence, but usually, only one or two biotic factors are often involved in most of the studies on this topic. In addition, plants often encounter a variety of attackers simultaneously or successively in nature. Such indirect interactions could be greatly distinct with the increase of pest species present in the same host. Nevertheless, there is no study on the effect of pest biodiversity, as the same important (probably more) as other biotic factors, on these indirect interactions. Also, another important abiotic factor, sublethal effects of pesticides, which is often linked to pest resistance and pest resurgence in the field, may serve as a major external force to shape the construction of pest communities on one host, but few are studied.

Thus, in my Ph.D. study, all the five biotic factors, including different feeding modes (chewing caterpillars, sap-feeding aphids, fungi and root-knot nematodes), host specialization (specialist/ generalist), attacking location (aboveground/ belowground) and attacking sequence

(sequentially/ simultaneously), pest diversity (1-4 pest species) and a new abiotic factor (sublethal effects of pesticides) are involved to evaluate their influences on the plant-mediated indirect interactions. Especially, pest biodiversity was first involved to assess whether a general relationship between pest performance and the number of pest species could be obtained (**Obj. 1**).

Thereafter, the aphid population dynamic was monitored to evaluate the long-term effects of such indirect interactions. Moreover, the above-belowground indirect interaction was as well examined on the influence of the herbivore-natural enemy interaction in a tri-trophic system (**Obj. 2**).

Finally, plant metabolomics (including non-volatiles and volatiles) were analyzed to reveal the phytochemical mechanisms underlying the plant-mediated indirect interactions and the tri-trophic interactions (**Obj. 3**).

Objectives

The objectives in my Ph.D. studies were to solve the questions as follows:

- 1. Which biotic and abiotic factors affect the plant-mediated indirect interactions?**
- 2. How the plant-mediated (above-belowground) indirect interactions impact the aphid population dynamic in a tri-trophic system?**
- 3. What are the phytochemical mechanisms underlying plant-mediated indirect interactions among multiple bioaggressors, and the tri-trophic interactions among plants, pests and natural enemies?**

CHAPTER 2. BIOLOGICAL MODEL: A TRI-TROPHIC AGROECOSYSTEM

Crops (first trophic level), agricultural pests (second trophic level) and natural enemies (third trophic level) are the basic components of the agroecosystem food web. The agricultural pests (including a variety of organisms, such as viruses, herbivorous insects, plant pathogens, root-knot nematodes) directly consume crops as their nutrient resources, but they are also under the threat to be attacked by the third trophic level (e.g. carnivorous insects). All three trophic levels interact with each other and co-evolve in nature. In this Chapter, the biological organisms involved in my study are introduced, including two major food crops (tomatoes and soybeans), four different feeding guilds of pests (chewing insects, sap-feeding insects, pathogenic fungi and root-knot nematodes), and one kind of natural enemy (parasitoids). In my Ph.D. study, tomato agroecosystem was the main studied system (Article 1, 2 in Chapter 3 and Chapter 4, 5). The soybean system was chosen for the Article 3 as an additional study.

1. Food crops

Food crops as an essential part of the diet of humans consist of grains, legumes, seeds and nuts, vegetables, fruit, herbs and spices, beverage plants, and so on. Legume family (Fabaceae) and nightshade family (Solanaceae) are two of the most important crops in agricultural production and the food industry due to their rich nutrient of proteins, carbohydrates, and vitamins. Tomatoes and soybeans as the major representatives in Fabaceae and Solanaceae respectively are widely cultivated in both Europe and all over the world. Therefore, the two species of crops will be mainly investigated in my Ph.D. study.

1.1. Tomato

The tomato, *Solanum lycopersicum* L. Solanaceae, native from the Peru-Ecuador area, is an important vegetable crop globally consumed and widely cultivated from tropical to the temperate zone (Jenkins, 1948; Sheaffer and Moncada, 2012). In spite of a tropical crop, tomato is grown in almost every corner all over the world and it is planted not only in the open field but also under greenhouses. It ranks first among vegetables and fruits as an important source of vitamin A and C, essential minerals as well as phenolic antioxidants (Sheaffer and Moncada, 2012). Additionally, it is one of the commercially important products throughout the world for consumption as fresh fruits and the food processing industry. The cultivated area of tomato all over the world was about 5 million hectares and the annual yield amounts to approximately 160 million tons (Knapp and Peralta, 2016). Moreover, approximately a quarter of the tomato

production is used for the processing industry and becomes the leading vegetable for processing in the world (Fig. 1).

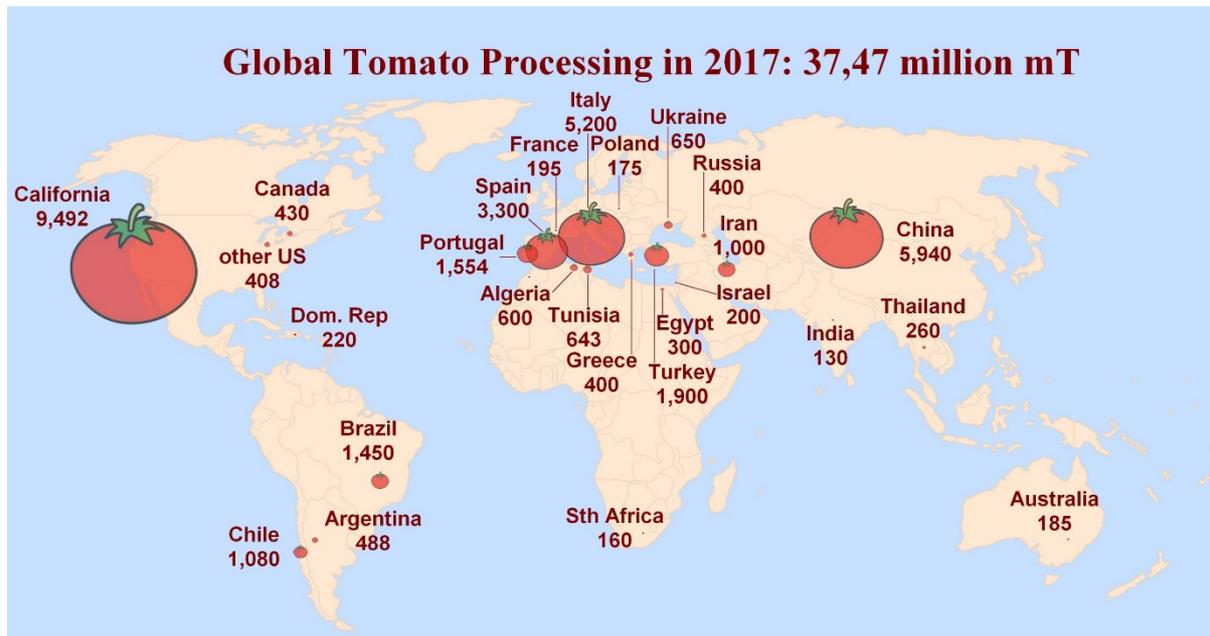


Figure 1. The global tomato processing industry in 2017 (<http://www.tomatonews.com>)

Additionally, tomato is also one of the most-studied dicotyledonous plants at the molecular and hormone metabolism levels (Arie et al., 2007; De Vos et al., 2018). It has been widely used as a model species for the studies on gene characterization for herbivore or pathogen resistance and the studies on phytohormonal functions in plant defensive pathways (Chetelat and Ji, 2006; Ji and Scott, 2006). Moreover, Solanaceae plants are known for producing glycoalkaloids (secondary metabolites) which function as natural defensive substances against insects and pathogens (Chowański et al., 2016). Tomato, as one species of this family, produces the spirosolane-type glycoalkaloids, α -tomatine and tomatidine (dehydrotomatine), to protect from insect pests and plant pathogens (Chowański et al., 2016).

1.2. Soybean

The soybean *Glycine max* L. Fabaceae, originated from China, has been cultivated in almost all the climatic areas in the world as one of the most valuable agriculture crops (Liu et al., 2008). It is a valuable and nutritious crop that is produced as both vegetable oil and supplemental protein source for human consumption (Van and McHale, 2017). Additionally, it has been widely used as the processing food and protein drinks due to its abundant high-quality dietary proteins. The crude protein content in the dry biomass of soybean ranges from 41% to 50% (Masuda and Goldsmith, 2008). Since the 1970s, the plantation area of soybean

crop increased remarkably from 29.5 million ha in 1970 to 117.5 million ha in 2014 (Food and Agriculture Organization, 2017). Response to the global increase in demand for soybean products in the past thirty years (Fig. 2), soybean has been the highest increasing crop in the percentage of the world's arable land compared to any other major crops.

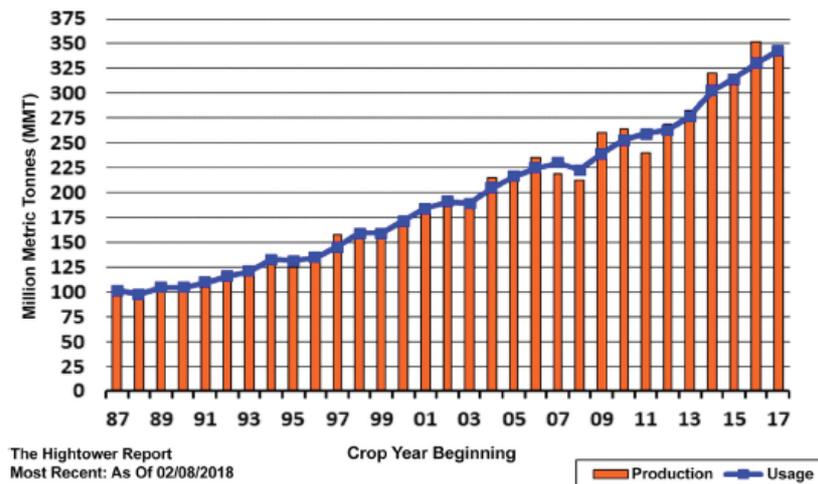


Figure 2. The global production and usage of soybean in the past three decades (<https://www.soymeal.org/soy-meal-articles/world-soybean-production/>)

Soybean plants are commonly challenged by a variety of biotic and abiotic stresses, such as herbivorous insects, nematodes, viruses, pathogens, salt and drought (Elhady et al., 2018). So far, although several strategies, involving selection of resistant species and introduction of natural enemies, have been developed as the sustainable approaches to control pests on soybeans, pesticide application still play a key role in rapid pest control in most countries (Desneux and O'Neil, 2008; Desneux et al., 2007, 2009; Koch et al., 2018; Qu et al., 2015).

2. Pest organisms

In this context, 'pests' indicate all the biotic aggressors that can attack plants, including various herbivores and phytopathogens. They contain a variety of different organisms. They can directly consume plants by chewing/defoliation, sucking plant sap, feeding cell content, or cause plant diseases. In addition, both the aboveground parts including leaves, stems, flowers and fruits, and the belowground roots can be attacked by pests. Some of them just feed on a specific species of plants, and other pests can colonize a wide range of plants (Bebber et al., 2013; Oerke, 2006).

2.1. Herbivorous insects

2.1.1. Sap-feeding aphids

Aphids (Hemiptera: Aphididae) specialized in feeding plant sap, are one of the most destructive pests on numerous crops (Giordanengo et al., 2010; Goggin et al., 2001; Hohenstein et al., 2019). They can rapidly achieve high population densities on account of their parthenogenesis reproduction and the short generation time. However, sexual reproduction is employed by aphids mostly for overwintering (Fig. 3). Alate aphids are usually produced in response to adverse conditions, e.g. overcrowding, poor plant condition, overwintering, or migration (Müller et al., 2001). Moreover, both wingless (apterae) and winged (alate) individuals of the same aphid species may be simultaneously on the same plants. Aphids can colonize almost all the crop organs leading to the vast consumption of photoassimilates, as sap-feeder. It may cause leaf chlorosis, defoliation, and necrosis, by directly sucking plant sap (Goggin et al., 2001). Additionally, they can also result in multiple viral diseases via transmission of plant viruses. Both the direct and indirect damage of aphids will negatively affect crop development and cause significant quality decline and yield loss (Giordanengo et al., 2010; Nalam et al., 2019; Powell et al., 2006).

Macrosiphum euphorbiae

The potato aphid, *Macrosiphum euphorbiae* Thomas, is a generalist pest with a wide host range including several plants in the Solanaceae (Teixeira et al., 2016). For instance, *M. euphorbiae* is a serious pest on processing tomatoes, *S. lycopersicum* L. and can cause significant quality and yield losses of the fresh tomato fruits by both direct and indirect damage (Goggin et al., 2001). Moreover, the potato aphid can also indirectly damage tomato plants by transmission of phytopathogenic viruses and promotion of sooty mold on the leaves, ultimately resulting in the yield loss of tomatoes (Lange and Bronson, 1981, Walgenbach, 1997). As reported in California, it caused serious yield loss of 1 ton per acre by heavy potato aphid infestations on the susceptible tomato varieties (Zalom et al., 1999).

Aphis glycines

The soybean aphid, *Aphis glycines* Matsumura, one of the specialist pests on soybean plants, has become a global pest in the last two decades (Koch et al., 2018; Kucharik et al., 2016). Native to Asia, the soybean aphid has been a major source of economic loss in soybean production in one of the major production areas (North America) since it was first documented in 2000 (Ragsdale et al., 2004). This invasive species colonized soybeans and rapidly spread

all over the north of America and south of Canada (Ragsdale et al., 2011). *A. glycines* can cause serious yield loss on soybeans by up to 40% - 58% via the reduced quantity of soybean pods, lessening seed size and wilting the entire soybean seedlings (Qu et al., 2017; Ragsdale et al., 2007). In addition to the direct consumption of plant sap, the soybean aphid can also act as vector of multiple viruses such as *Soybean mosaic virus (SMV)*, and *Alfalfa mosaic virus (AMV)*, and even potentially contribute to the infestation of soybean cyst nematodes in soybean roots (Hill et al., 2001; Kucharik et al., 2016).

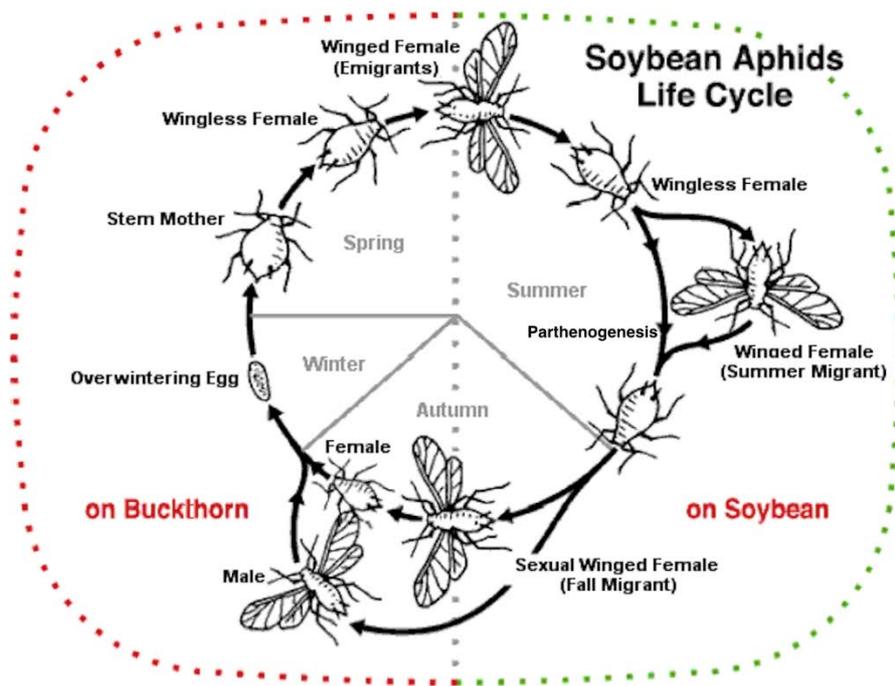


Figure 3. The life cycle of the soybean aphid (from Wang et al., 1962).

Aulacorthum solani

The foxglove aphid, *Aulacorthum solani* Kaltenbach, native to Europe, has been a cosmopolitan pest feeding on a wide range of host plants. It can colonize approximately 95 different plant species from 25 families, e.g. pepper, potato, lettuce and soybean (Down et al., 1996; Jandricic et al., 2014; Sanchez et al., 2007, Fig. 4b, 4c). For example, *A. solani* could cause leaf necrosis, deformation of soybean grains, and even defoliation of the entire plants at high density due to the toxicity of its salivary secretions to soybean plants (Jandricic, 2013; Sanchez et al., 2007). Moreover, the *Soybean dwarf virus (SDV)* can be transmitted by *A. solani* resulting in viral disease in soybean (Jandricic, 2013, 2014). Furthermore, this aphid benefits the fungal growth on leaves and thereby reduces crop photosynthesis (Sato et al., 2013, 2014).

All these problems may cause significant yield loss in the soybean crop up to 70-90% (Nagano et al., 2001; Sato et al., 2013).

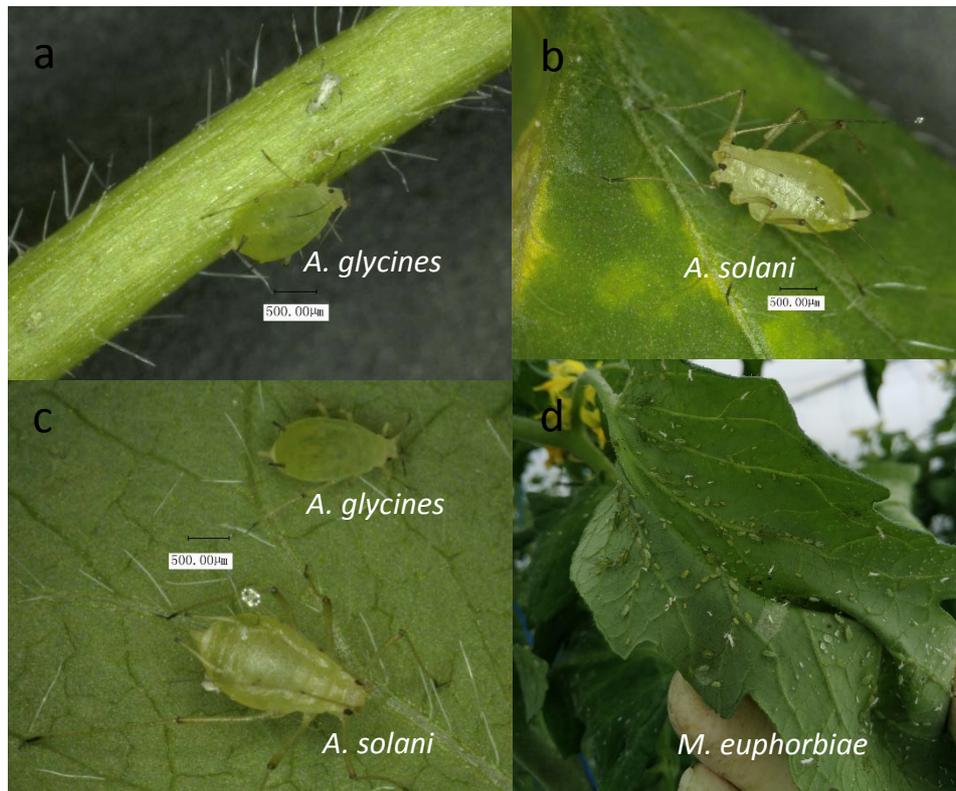


Figure 4. Aphids used in the trials.

2.1.2. Chewing caterpillars

The caterpillars are the larval stages of Lepidopterous moths (Fig. 5) and they are able to chew most plant tissues, including roots, young stems, leaves, flowers, and fruits with their powerful jaws. The complete defoliation may be achieved when the host is particularly favored by the caterpillar. At the beginning of the lifecycle (Fig. 5), eggs may be laid singly or in small groups. The newly hatched larvae initially eat their own egg cases before feeding on plant tissues. Thereafter, they start to massively feed on almost all the crop organs during their larval stages. In the last instar of larvae, the caterpillars stop eating and move in preparation for pupation. Adults emerge from pupae and lay eggs in an adequate place after mating. The development and oviposition of caterpillars are temperature-dependent and photoperiod-dependent as well as most insects (Patil et al., 2017). For instance, at a mean temperature of 28 °C, it takes about 30-34 days for cotton bollworm to develop from eggs to adults (Zalucki et al., 1986; Fig. 5).

Helicoverpa armigera

The cotton bollworm *Helicoverpa armigera* Hübner is a polyphagous pest that can feed on a wide range of economic crops. There are more than 180 plant species reported as hosts of *H. armigera*, including tomato, cotton, pigeon pea, chickpea, sorghum, cowpea, field beans, soybeans, tobacco, potatoes, maize, and a number of vegetable and flower crops (Gahukar, 2002; Kakimoto et al., 2003; Patil et al., 2017). To date, it has been one of the most notoriously agricultural pests and widely distributes in Australia, Asia, Europe and Africa (Tay et al., 2013). The pest is a fruit borer of tomatoes and also prefers to feed on the floral bodies of the host (Arora et al. 2011; Dalal and Arora, 2016). In tomato, it can cause yield loss by up to 70% due to leaves chewing and fruit boring (Sharma, 2001). Furthermore, it is estimated that annually global economic losses caused by *H. armigera* alone are about 5 billion dollars (Sharma, 2001).

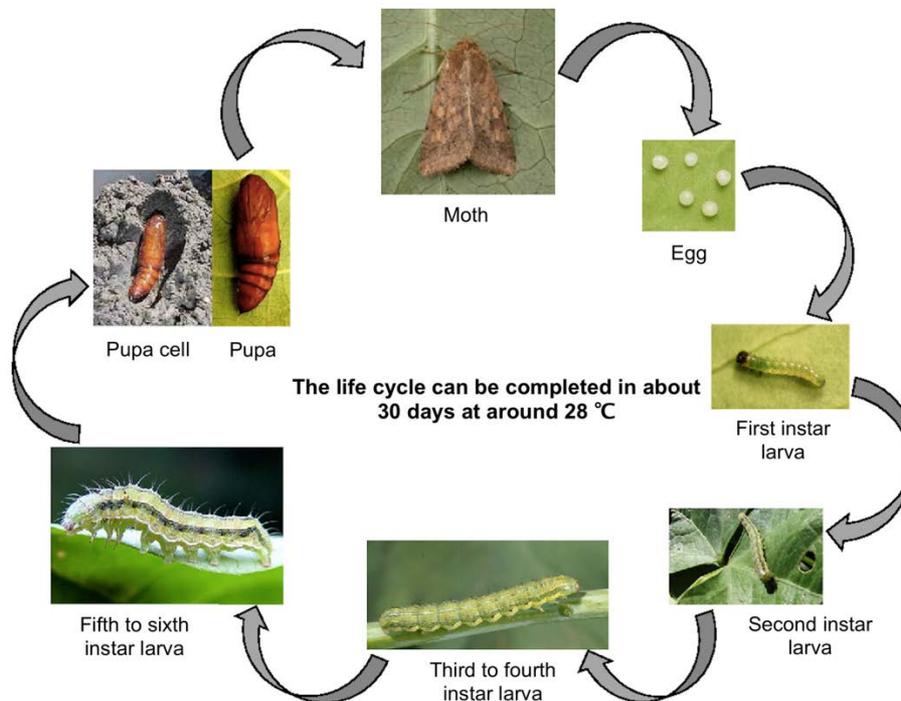


Figure 5. The life cycle of *H. armigera* (from Patil et al., 2017)

Spodoptera exigua

The beet armyworm, *Spodoptera exigua* Hübner, is a polyphagous insect that infests numerous crops including chili, clover, cotton, potato, soybean, tomato, and many vegetable, flowers, and weed species (De Luna-Santillana et al., 2011; Zhang, 2009). It originates from Southeast Asia and has been a cosmopolitan invasive pest on various agricultural crops (Saeed et al., 2010). The distribution of this pest has increased to 101 countries in the tropical and subtropical regions, such as Africa, southern Europe, Asia, South and North America (Zheng et al., 2011). The *S. exigua* larvae feed on both the foliage and fruit of plants, and can even defoliate the

entire young seedlings. Young larvae usually hide and feed on the undersurface of leaves and feed on there and they do not cause obvious chewing symptoms on the upper epidermis. When developing to larger larvae, *S. exigua* caterpillars make irregular holes in leaves and even devour the foliage completely (An et al., 2016). On tomato plants, both growing points, buds, and fruits can be attacked by the larvae of *S. exigua*, resulting in growth stagnation and yield decline (Taylor and Riley, 2008).

2.2. Phytopathogenic organisms

plant pathogens include all the biotic organisms which cause plant disease and may end with the death, such as viruses, bacteria, fungi, oomycetes, nematodes, etc... (Bruehl, 1991)

2.2.1. Fungal plant pathogen

Oidium neolycopersici

Tomato powdery mildew caused by plant pathogenic fungus, *Oidium neolycopersici* L. Kiss, is one of the most devastating diseases of cultivated tomatoes and has emerged in many countries involving in Europe, Asia, Africa, North and South America for the last three decades (Blancard, 2012). *O. neolycopersici* is an epiphytic biotrophic fungus and caused seriously epidemic infections on tomato crops both in protected greenhouses and open fields (Jones et al., 2001, Mieslerová et al., 2002). Additionally, spores of this fungi can quickly reside on tomato leaves by the rapid reproduction and spread capacity (Fig. 7), which makes a greater challenge to manage this fungal pest. Moreover, most tomato cultivars are considered highly susceptible to this powdery mildew fungus (Lebeda and Mieslerová, 2002). A few white and circular powdery spots can be observed at the tip of the infested leaflet after the tomato is attacked by powdery mildew spores (Fig. 6a). More isolated white powdery colonies are scattered over the upper side of the leaves due to the spore spread with airflow (Fig. 6b; 6d). The stem of the tomato plant can be colonized by *O. neolycopersici* as well (Fig. 6c). However, the colonies of such fungi are seldom found on tomato fruits. Thereafter, fungal patches cluster to cover the whole leaflet (Fig. 6e) and result in yellowing of the leaflet (Fig. 6f). Ultimately, it can cause the entire tomato seedling wilting under a heavy infestation of tomato powdery mildew (Fig. 6g).

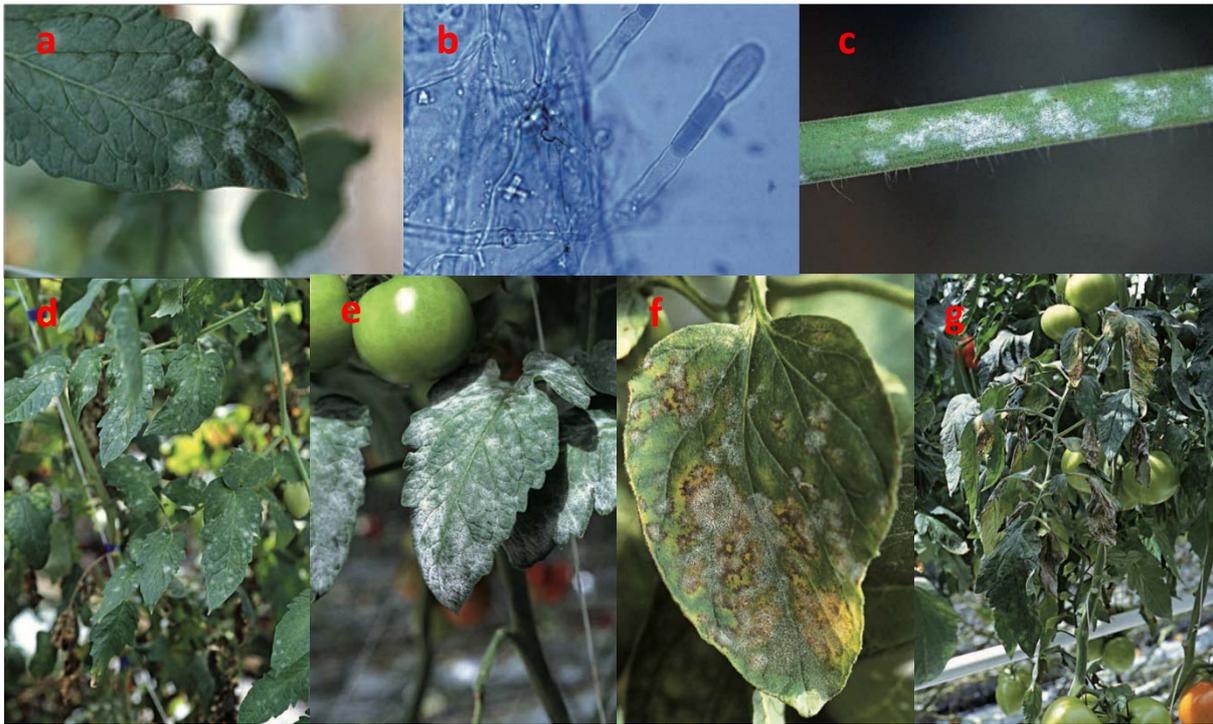


Figure 6. Infestation of *O. neolycopersici* on tomato plants. a): several white and circular powdery spots are visible at the tip of the leaflet at the initial infestation stage; b): *O. neolycopersici* produces mainly single ellipsoidal to ovoid hyaline conidia to colonize the upper surface of tomato leaves; c): the stem of tomato plants can be also colonized by *O. neolycopersici*; d): more isolated white powdery colonies are scattered over the tomato leaves via spore spread with airflow; e): the colonies of *O. neolycopersici* converge and thereafter cover the entire leaflet; f): the leaflet is increasing yellow; g): *O. neolycopersici* inexorably colonizes this tomato plant, and the infested leaves are partially necrotic and eventually completely dry (from Blancard, 2012).

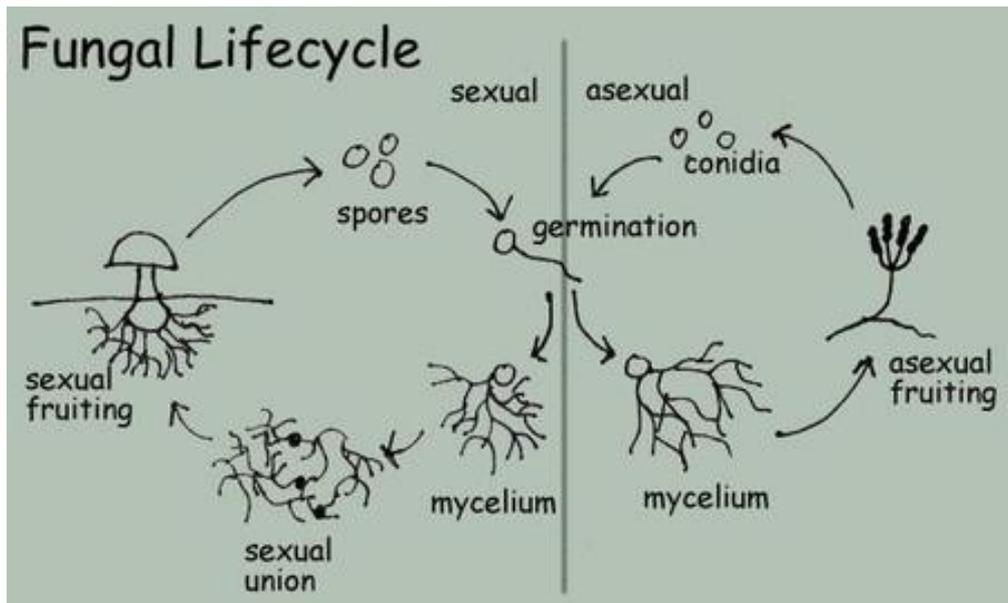


Figure 7. The life cycle of fungus (http://www.tanelorn.us/data/mycology/myc_life.htm.)

Fungi can be reproduced in both sexual and asexual ways. Generally, fungi begin their lives as a spore, then germinate and develop into a web, which is called as mycelium. Especially, many fungi can produce asexual fruiting bodies that bear asexual spores (conidia). Conidia are often generated quickly and easily, which contribute to the fast occupation on their host.

2.2.2. Root-knot nematodes

Meloidogyne incognita

The southern root-knot nematode, *Meloidogyne incognita* Kofoid and White, one of the plant-parasite nematodes, is ubiquitous in distribution and can infect more than 1700 species of crops, especially vegetables, including tomatoes (Ji et al., 2019; Karuri et al., 2017; Mukhtar et al., 2017). It is one of the most disastrous and prevalent threats to crops due to its damaging infection of crop roots and results in significant yield loss in most warm climates (Khan et al., 2017; Kiewnick and Sikora, 2006). *M. incognita* can maintain a relationship with its host for 3-8 weeks (Fig. 8). The infective second-stage juvenile (J2) hatches in the soils, penetrates a root tip, and then migrates intercellularly through the root cortical tissue to the differentiation zone of vascular cylinder. There the nematode becomes sedentary and injects secretory proteins to induce the generation of a permanent feeding site. The feeding site was formed with enlarged and multinucleated cells as well as known as a giant cell (Abad et al., 2009; Caillaud et al., 2008). The giant cell provides necessary nutrients to the nematode for its development but the root tissue surrounding the feeding site undergoes hyperplasia thereby causing root galls or root knots. The nematode continues to develop into the third-stage juvenile (J3), fourth-stage

juvenile (J4) and finally into the adult (Moens et al. 2009). The vermiform male leaves the root but the female deposits eggs in a gelatinous matrix (called egg mass) out of the gall surface after continuous grow until a pear shape (Manzanilla-Lopez et al., 2004). The first-stage juvenile (J1) develops inside the egg and thereafter J2 hatches from the egg to migrate towards a new host root (Dubreuil et al., 2011; Martinuz et al., 2013).

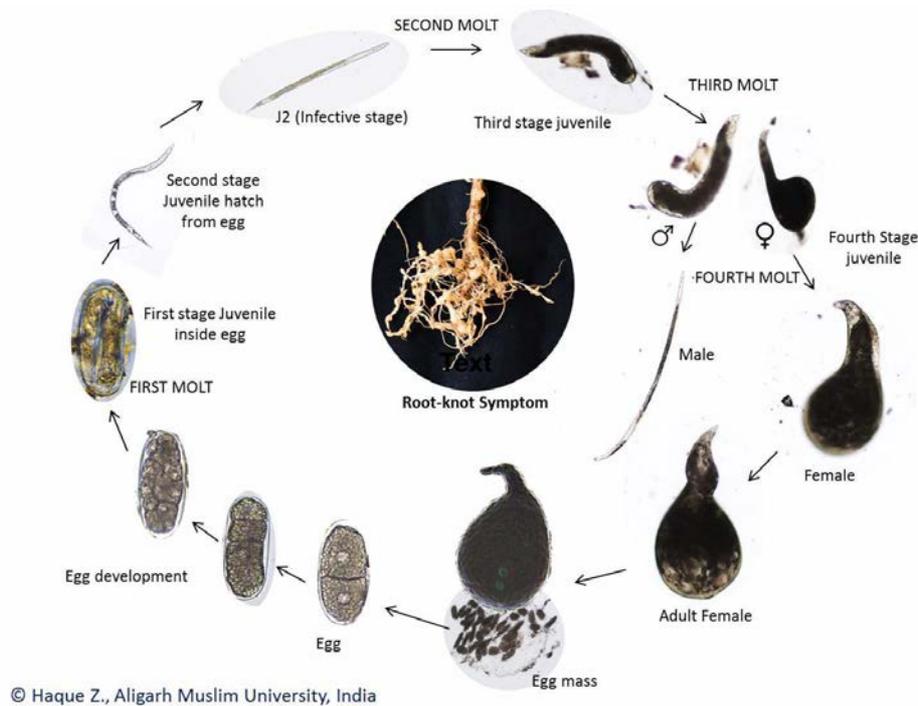


Figure 8. The life cycle of root knot nematode, *Meloidogyne* spp. (from Ziaul Haque, Aligarh Muslim University, India, 2017)

3. Natural enemy

Parasitoids (Aphidius ervi)

Aphidius ervi Haliday (Hymenoptera: Braconidae) originates from Europe and now has been introduced into Asia, Australia, North and South America, and many other countries (Velasco-Hernández et al., 2017). It can parasitize many aphid species but it is most commonly used to control larger aphid species such as *M. euphorbiae* (the potato aphid), *A. solani* (the foxglove aphid), or *Acrythosiphum Pisum*. (the pea aphids) (Helyer et al., 2014). To date, *A. ervi* has been one of the biocontrol agents commercially produced by a number of biocontrol companies and commonly used in the control of *M. euphorbiae* in agrosystem (Helyer et al., 2014).

Each female adult can lay around 100 eggs (one per aphid individuals) in aphids and aphids keep on moving and feeding after the egg of *A. ervi* has been deposited into their bodies. The

A. ervi larva develops entirely inside the aphid after the parasitoid egg hatches (Fig. 9), and eventually kill the host when the wasp larva is ready to pupate. The parasitoid larva pupates within the aphid body, which results in a rigid and golden-brown mummy. The *A. ervi* adult emerges by chewing a hole through the back of the mummy and leaves to seek out new hosts. Complete development time depends on the temperature and it is around 19 days at 21°C (Fig. 9). Even though this is longer than the development time of aphids, each *A. ervi* female can attack more than 300 aphids in the process of hunting and survive 2-3 weeks with adequate food and water. Initially, the aphid control by *A. ervi* is slow. However, as long as a certain degree of damage can be tolerated, it is possible to achieve a high and even entire control of aphids. The efficacy of the parasite may be reduced when the temperature is above 30°C or below 8°C (Henry et al., 2005; Lenaerts et al., 2017).

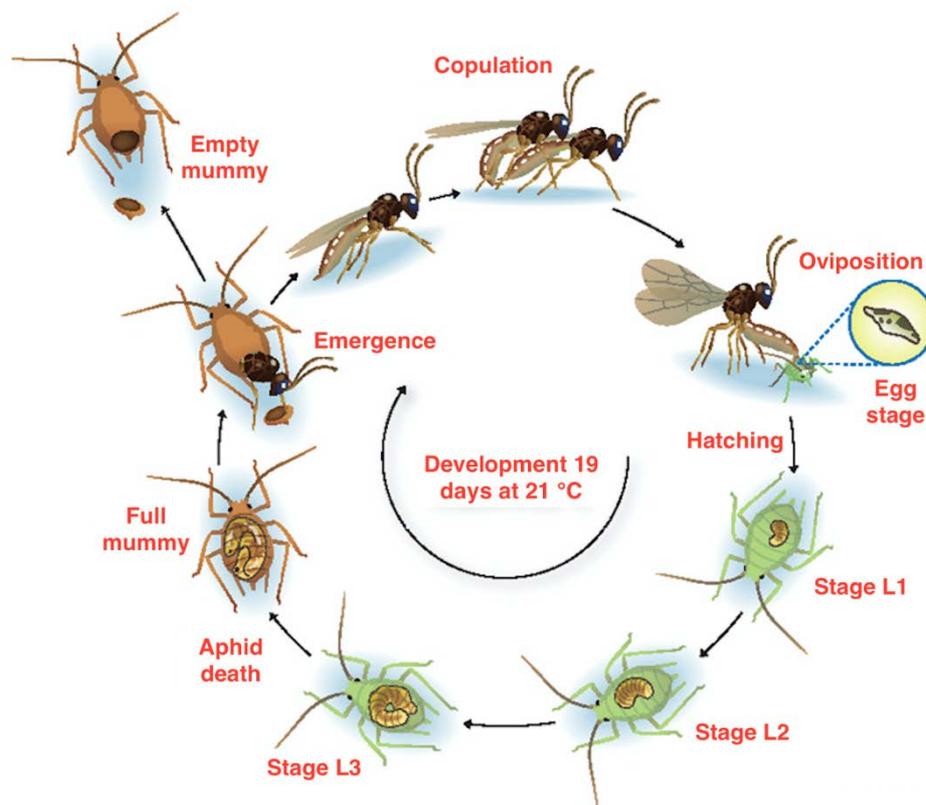


Figure 9. The life cycle of *A. ervi*. (from Bernard Chaubet, INRA, France, 2018)

In my Ph.D. study, all the organisms described above are used.

Firstly, sap-feeding aphids (*M. euphorbiae*), chewing caterpillars (*H. armigera* /*S. exigua*), powdery mildew fungi (*O. neolycopersici*), and root-knot nematodes (*M. incognita*) were used to assess multiple biotic factors on plant-mediated indirect interactions on tomatoes (Article 1 and Article 2 in Chapter 3).

Secondly, the specialist aphid (*A. glycines*) and the generalist aphid (*A. solani*) are together introduced on soybeans to evaluate the sublethal effects of pesticides on their interspecific interactions (Article 3 in Chapter 3).

Thirdly, root-knot nematodes (*M. incognita*), fungi (*O. neolycopersici*), aphids (*M. euphorbiae*) and parasitoids (*A. ervi*) were all introduced on tomato to assess the influence of above-belowground interactions among the three pest species on the aphid population dynamics and the natural enemies (Chapter 4).

CHAPTER 3. BIOTIC AND ABIOTIC FACTORS MODULATE PLANT-MEDIATED INDIRECT INTERACTIONS

Global food security is threatened by the emergence and spread of crop pests and pathogens. Spread is facilitated primarily by human transportation, but there is increasing concern that climate change allows establishment in hitherto unsuitable regions (Bebber et al., 2013). Whether through deliberate or accidental introduction, many natural environments now host assemblages of exotic species that threaten populations of native species (McGeoch et al., 2010) and alter ecosystem function (Clavel et al., 2010).

In nature, plants are commonly confronted with various biotic stresses, including herbivorous insects, fungi, bacteria, nematodes and viruses. These pests can attack plants both simultaneously or sequentially. The plant-pest interactions can cause chemical alterations of the plant, consequently leading to indirect interactions among multiple organisms which share the same host. Such indirect interactions can be modulated by both biotic and abiotic factors. In this chapter, the modulation of **five biotic factors (pest biodiversity, feeding strategy, attacking sequence, attacking location and host specialization as described in Chapter 1), and one abiotic factor (sublethal effects of pesticides)** were assessed on the plant-mediated indirect interactions. **Pest biodiversity, feeding strategy, attacking sequence, attacking location were assessed in Article 1 and Article 2, and host specialization as well as sublethal effects of pesticides was assessed in Article 3.**

In Article 1, the impact of pest biodiversity on plant-mediated indirect interactions was assessed under a simultaneous attack of the host plant. Four pests with different species abundance and feeding strategies, i.e. the piercing-sucking aphid, the chewing caterpillar, the plant pathogen and the root-knot nematode, were introduced simultaneously on tomato plants, *Solanum lycopersicum* to evaluate the plant-mediated indirect interactions among these pests. Nematodes attacked tomato roots and the other three pests attacked the tomato leaves. **A general correlation was observed between insect performances (aphid population and development rate of caterpillar larva) and the number of pest species involved in the plant-mediated indirect interactions while such the relationship was not detected on pathogenic organism performances (fungal reproduction and invasion rate of nematodes). Insects benefited from the increase of pest species number in the indirect interactions when the host was infested by other consumers at the same time. In addition, the indirect interactions between aboveground pests and belowground pests are unidirectional when 2 pests attacked tomato plants at the same time. Namely, nematodes (the only**

belowground pest) were affected by the aboveground pest, while the aboveground pests were not impacted by nematodes. However, the bidirectional interactions were observed when 3 pests infested simultaneously the same host, i.e. both nematodes (the only belowground pest) and aphids (the aboveground pest) were affected in the 3-pest interactions (nematodes+ fungi+ aphids). Therefore, the directions of plant-mediated indirect interactions between aboveground and belowground pests relied on the species number involved in the indirect interactions, when pests attacked the host plant at the same time.

In Article 2, the same modulation factors were evaluated when tomato plants were infested by multiple pests in sequence. The same 4 chosen pests were introduced sequentially on tomato plants. Nematodes, fungi, aphids and caterpillars are introduced on tomato plants in sequence. In this article, the insect performances were negatively correlated to the species number involved in plant-mediated indirect interactions but the plant growth in plant height and stem nodes was positively correlated to the species number involved in indirect interactions when pests arrived on the host in sequence. Similarly than in Article 1, there was no correlation between pathogenic organism performances and the pest species number involved in indirect interactions. Nematodes, i.e. the only belowground pest and also the first arrived pest in the sequence, unidirectionally affected the aboveground pests and the subsequently arriving pests when they attacked the host sequentially.

In Article 3, Beta-cypermethrin, one of the pyrethroid pesticides with a broad of insecticide spectrum, has been widely used to control aphids in the soybean fields depending on its high efficacy and long persistence with a stable formation (Hodgson et al., 2012; Ragsdale et al., 2011). It has been detected in my previous study that sublethal concentration of beta-cypermethrin stimulate the reproduction of soybean aphids (Qu et al., 2017). Moreover, the sublethal effects of pesticides are often associated with the pest resurgence in the field after the application of pesticides (Dutcher, 2007). Sublethal concentration of beta-cypermethrin, as an important abiotic factor, was therefore assessed in a context of interspecific interactions between the specialist aphid (*Aphis glycines*) and the generalist aphid (*Aulacorthum solani*) on the soybean, by measuring aphid life-history traits and host susceptibility. The specialist aphid had better performance (higher fecundity) on soybeans. Sublethal concentration of beta-cypermethrin stimulated the reproduction of *A. glycines*, but it did not alter the fecundity of *A. solani* at this concentration. When *A. glycines* and *A. solani* attacked soybean plants in sequence, the fecundity of the subsequent attackers on the soybean plant was inhibited by the interspecific interactions. Additionally, interspecific interactions caused stronger

suppression on the reproduction of the specialist aphid *A. glycines*, compared to the generalist aphid *A. solani* on soybeans. Colonization of the subsequent aphids on the host benefited from the pre-infestation by the other aphid species via the induced plant susceptibility. However, exposure to the sublethal beta-cypermethrin caused a stronger interspecific suppression on the fecundity of the two species of aphids, but it impaired the colonization facilitation caused by hetero-specific aphids to subsequently arriving aphid species.

In conclusion, in tomato system, a general relationship between insect performances and the pest species number involved in the indirect interactions was observed, whether pests attacked tomato plants simultaneously or sequentially. Whereas, there was not a correlation between pathogenic organism performances and the pest species numbers in indirect interactions. When pests attacked tomato plants at the same time, insect performances were positively correlated with the pest species number in indirect interactions. However, when pests attacked tomato plants in sequence, insect performances were negatively correlated with the pest species number in indirect interactions. Additionally, plant fitness was positively correlated with the pest species number in indirect interactions. **The interactions between aboveground pests and the only belowground pest (nematodes) as well depend on attacking sequence and pest biodiversity.** The aboveground pests were unidirectionally affected by the belowground nematodes, when they attacked tomatoes in sequence. However, when pests simultaneously attacked tomatoes, the above-belowground interactions were dependent on the pest species number on the same plant. When 2 pests attacked tomatoes at the same time, belowground nematodes were unidirectionally impacted by the aboveground pests, but nematodes and aboveground pests affected each other under three-pest interactions. **Furthermore, whether nematodes, fungi and aphids attacked the same tomato plant simultaneously or sequentially, the number of aphid individuals were reduced by the plant-mediated indirect interactions.**

In soybean system, exposure to sublethal concentration of beta-cypermethrin accelerated the interspecific competition between the specialist and the generalist aphids, and impaired the induced susceptibility of host to the interspecific aphids.

Chapter 3. Biotic and abiotic factors modulate plant-mediated indirect interactions

Article 1

Qu YY, Lavoit AV, Bearez P, Castagnone-Sereno P, Nicot PC, Monticelli LS, Desneux N

Impact of biodiversity and feeding guilds on plant-mediated indirect interactions linking aboveground and belowground pests

Journal of Pest Science, submitted

Article 2

Qu YY, Lavoit AV, Amiens-Desneux E, Monticelli LS, Wang YS, Castagnone-Sereno P, Nicot PC, Desneux N

Impact of biodiversity on plant-mediated indirect interactions under sequential pest infestation.

In preparation

Article 3

Qu YY, Ullah F, Luo C, Monticelli LS, Lavoit AV, Gao XW, Song DL, Desneux N

Sublethal effects of beta-cypermethrin modulate interspecific interactions between the specialist aphid *Aphis glycines* and the generalist aphid *Aulacorthum solani* on soybeans

Ecotoxicology and Environmental Safety, accepted

1. Impact of biodiversity on plant-mediated indirect interactions under simultaneous pest infestation

Article 1

Qu YY, Lavoit AV, Bearez P, Castagnone-Sereno P, Nicot PC, Monticelli LS, Desneux N

Impact of biodiversity and feeding guilds on plant-mediated indirect interactions linking aboveground and belowground pests

Journal of Pest Science, submitted

16 **Abstract**

17 Plant-mediated indirect interactions which largely depend on systemically induced defenses on plants
18 could connect diverse organisms occupying different spatial niches on the host. In the current study, pests
19 with different species abundance and feeding strategies, i.e. the piercing-sucking aphid (*Macrosiphum*
20 *euphorbiae*), the chewing caterpillar (*Spodoptera exigua*), the plant pathogen (*Oidium neolycopersici*)
21 and the root-knot nematode (*Meloidogyne incognita*), were introduced simultaneously on tomato plants,
22 *Solanum lycopersicum* to evaluate the plant-mediated indirect interactions among these pests. When two
23 different feeding guilds of pests attacked the host simultaneously, the aboveground attackers significantly
24 impacted both aboveground and belowground pests, i.e. aphids and powdery mildews increasing
25 nematode infection rate on tomato roots; the aphid population was suppressed due to the presence of beet
26 armyworms or powdery mildews on the tomato plants. When three different attackers shared the host,
27 such indirect interactions between aboveground and belowground attackers are bidirectional. The
28 infection rate of nematodes on roots was attenuated by the combined infestation of aphids and powdery
29 mildew on tomato plants. Nematodes working together with powdery mildews or beet armyworms also
30 impacted the aphid population negatively. Nevertheless, beet armyworms were the only members
31 benefitting from such interactions when four species of pests consumed the host. In particular, we found
32 the performance of insects, i.e. the aphid population and larval development of beet armyworm, were
33 positively correlated to the abundance of indirect interactions. However, such a correlation was not
34 detected between the life-history traits of phytopathogenic organisms, i.e. reproduction of powdery
35 mildew fungi and nematode galls, and indirect interaction abundance.

36 **Key words:** plant-mediated interactions, induced plant defense, pest biodiversity, feeding guilds,
37 aboveground-belowground interactions

38 **Key message**

39 ● Plant-mediated indirect interactions linking aboveground and belowground pests were firstly
40 studied from the perspective of biodiversity involving four feeding guilds of pests.

41 ● The above-belowground interactions were unidirectional when two pests attacked tomato
42 simultaneously, i.e. aboveground pests significantly affected the performance of belowground
43 nematodes, rather than vice versa. However, such interactions were bidirectional when three
44 pests shared the same host. Moreover, beet armyworms were the only members benefitting from
45 such interactions when four species of pests consumed the host.

46 ● A general relationship was obtained that performance of insects, i.e. aphid and beet armyworm,
47 was positively correlated to the increasing abundance of indirect interactions while such a
48 correlation was not suitable for phytopathogenic organisms, i.e. powdery mildew fungi and
49 nematodes.

50

51

52 **Introduction**

53 Plant defenses against herbivorous pests involve not only constitutive defenses but also induced defenses
54 including local and/or systemic syntheses of secondary metabolites, as well as the indirect defenses by
55 releasing volatiles to attract natural enemies (Dangl & Jones, 2001; Gatehouse, 2002; Wittstock &
56 Gershenzon, 2002; Dicke et al., 2003; Kaplan et al., 2008, Hatt et al., 2019). Consequently, physiological
57 traits of plants would change as a result of interactions between the plants and their consumers, which
58 may subsequently impact the behavior, survival, development and fecundity of other pests sharing the
59 same plant (Gatehouse, 2002; Mouttet et al., 2011). Such indirect interactions may have important
60 consequence for both plants and pests. On the one hand, interactions between different plant attackers
61 could entail an integrated defensive web which maximizes plant fitness in addition to simply synergistic
62 or antagonistic effects on plant performances (Agrawal, 2011; Hauser et al., 2013). On the other hand,
63 plant attackers have also developed different means to respond to and even to actively inhibit plant
64 defenses by manipulating host susceptibility to subsequent arrivals (Bouarab et al., 2002; Mouttet et al.,
65 2013; Schimmel et al., 2017). These indirect interactions may have effects not only on pest individuals
66 but also on the population dynamics in both managed and natural ecosystems (Stout et al., 2006; Poelman
67 et al., 2008; Stam et al., 2018).

68 Such indirect interactions among different plant attackers are largely influenced by the variations
69 in phytohormonal signaling pathways, mainly modulated by the following plant hormones: salicylic acid
70 (SA), jasmonic acid (JA) and ethylene (ET). These phytohormones can act as signaling molecules for
71 the production of an array of metabolites and the signaling pathways are specifically triggered by
72 different pest feeding guilds (Heidel & Baldwin 2004; Stout et al., 2006; Di et al., 2017). Although it is
73 observed that JA and SA signaling pathways antagonize each other, the cross-talk between these two

74 signaling pathways depends more on the dose, location and activated time of hormones (Leon-Reyes et
75 al., 2010; Thaler et al., 2012; Caarls et al., 2015). Moreover, resource reallocation and modulation of
76 source-sink relationships in plants also play key roles in such indirect interactions. Plants can divert
77 photosynthetic products from the injured tissues into other tissues that are inaccessible to phytophage
78 foraging and save them for re-use after herbivore attack (Erb et al., 2009; Lemoine et al., 2013; Kundu
79 et al., 2018).

80 Plant-mediated indirect interactions largely depending on induced defenses may connect pest
81 species that are both temporally and spatially separated. The arrival of the first pest induces plant
82 metabolite changes that subsequently impact preference and performance of the other organisms which
83 exploit the same plant, either simultaneously or sequentially (Mouttet et al., 2013; Biere & Goverse,
84 2016; Schimmel et al., 2018). For instance, a meta-analysis by Johnson et al. (2012) demonstrated that
85 belowground herbivores facilitate aboveground herbivore performance only when belowground and
86 aboveground pests simultaneously attacked a host plant. Furthermore, plant-mediated indirect
87 interactions between pests are not restricted to the same part, i.e. the shoot or root, of plants and they can
88 link belowground and aboveground parts via systemically induced plant defenses (Soler et al., 2012b,
89 2013; Wondafrash et al., 2013; Lee et al., 2017; Kundu et al., 2018). Pre-infestation by root knot
90 nematode *M. incognita* on tomato root noticeably suppresses whitefly population due to the systemic
91 acceleration of SA-dependent signaling pathway as well as decreased concentration of nitrogen in tomato
92 leaves (Guo & Ge, 2017). The foliar insect *Pieris brassicae* on *Brassica nigra* induced a lower survival
93 rate of root-feeding herbivore *Delia radicum* by more than 50%, which probably depends on the elevated
94 indole glucosinolates contents of roots (Soler et al., 2007). Such indirect interactions between
95 aboveground and belowground pests may be unidirectional or bidirectional and could lead to positive,

96 negative or neutral impact on pests. This account for numerous biotic and abiotic factors involving pest
97 attacking sequences, attacking locations (shoot or root), the magnitude of pest infestation, pest feeding
98 strategies, biodiversity, plant species, temperature, drought, nutrient and even pesticides (Staley et al.,
99 2007; Johnson et al., 2009, 2012; Ali & Agrawal, 2015; Barber et al., 2015; Kroes et al., 2016; Gaillard
100 et al., 2018).

101 In nature, it is common that the plant is infested by diverse pests including viruses, plant pathogens,
102 herbivorous insects and phytophagous nematodes successively or simultaneously (Soler et al. 2013; Ueda
103 et al., 2019). Undoubtedly, it becomes more difficult to predict the outcome of indirect interactions
104 between aboveground and belowground organisms, especially after different feeding strategies of pests
105 on their host with an abundant species diversity. As a consequence of the increase in pest species, cross-
106 organ defensive loops, i.e. shoot-root-shoot and/or root-shoot-root, are generated on plants as an
107 integrated defensive system connecting all the plant consumers and thereafter strongly affecting the pest
108 performance. However, so far, most of the indirect interactions among plant consumers were focused on
109 specific numbers of pest species, e.g. two or three pest species. The effect of plant-mediated indirect
110 interactions among more pest species (more than 3 species) with different feeding guilds and the effect
111 of the pest species number on such plant-mediated indirect interactions were scarcely studied. Therefore,
112 the impact of pest biodiversity involving four main feeding strategies of pests, i.e. *Macrosiphum*
113 *euphorbiae* (sap-feeding aphid), *Spodoptera exigua* (chewing caterpillar), *Oidium neolycopersici* (plant
114 pathogen) and *Meloidogyne incognita* (root-knot nematode) on plant-mediated interactions between
115 aboveground and belowground pests was investigated in this study. Moreover, we would like to look
116 into whether a general relationship exists between the pest performance and pest biodiversity when they
117 attack the host simultaneously. Not only may it provide a new way of forecasting or explaining

118 population dynamics of different crop pests in the ecosystem, but it may also facilitate greater
119 understanding of how the integrated plant defenses are manipulated to combat multiple biotic threats.

120

121 **Materials and methods**

122 *Study organisms*

123 Tomato plants, *Solanum lycopersicum*, Solanaceae (cultivar “San Marzano Nano”), were grown in a
124 climate controlled greenhouse (23±0.3°C in daytime, 17±0.2°C at night, RH: 64±1%, irradiance: 523±32
125 W/m²). They were planted in the substrate combination which contains soil and perlites and supplied
126 with a standard nutrient solution (pH=6, electrical conductivity (EC) =1.6 mS/cm, ion concentration: 620
127 mg/L NO₃⁻, 11 mg/L NH₄⁺, 170 mg/L H₂PO₄⁻, 115 mg/L SO₄²⁻, 180mg/L K⁺, 212 mg/L Ca²⁺, 12 mg/L
128 Mg²⁺).

129 The *M. incognita* nematode isolate used in the study came from the collection in INRA, Sophia Antipolis,
130 France. Infective second-stage juveniles (J2) were recovered from infested tomato plants grown under
131 greenhouse conditions according to the procedure of Neveu et al. (2003). The *O. neolycopersici* powdery
132 mildew fungi isolate was from a tomato culture in INRA, Avignon, France. Spores suspensions were
133 prepared by adequately blending the fully-infested tomato leaves with distilled water. The supernatant
134 was collected for the subsequent quantification of spore concentration which was measured with a
135 Mallasez[®] cell and adjusted to a final concentration of 10⁵ spores mL⁻¹. The *S. exigua* pupae were
136 provided by Dr. Salvador Herrero (University of Valencia, Valencia, Spain). The beet armyworm colony
137 was established by feeding them with artificial food in climatic chambers at INRA (L: D = 16:8, 24±1 °C,
138 55±5% RH). The colony of aphids, *M. euphorbiae*, was reared on the caged tomato plants at INRA,
139 Sophia Antipolis, France.

140

141 *Experimental design*

142 Fifteen treatments with different pest species numbering 1 to 4 was set up in this experiment, i.e. fifteen
143 different infestation treatments respectively including one, two, three or four pests were conducted on
144 tomato plants (Table 1). Each plant represented one replicate and each treatment was replicated 10 times.

145 The 5-week-old tomato seedlings (with five fully-developed leaves) in the climatic greenhouse
146 ($23\pm 0.3^{\circ}\text{C}$ in daytime, $17\pm 0.2^{\circ}\text{C}$ at night, RH: $64\pm 1\%$, irradiance: $523\pm 32\text{ W/m}^2$) were used for pest
147 consumption. All the different pests were introduced onto tomato plants simultaneously. A concentration
148 of 0.5 mL inoculum containing 500 J2 of *M. incognita* was inoculated into the soil around tomato roots.
149 The *O. neolycopersici* suspension of 10 μL was introduced onto one leaflet (the third fully-developed
150 leaf from the top of the tomato plant) and it was isolated in a Petri dish (diameter = 10cm, height = 7cm).
151 A 3cm diameter hole was dug on the cap and covered with nylon mesh, which facilitates moisture
152 reduction inside the dishes. Twenty third-instar aphids were settled on the second fully-developed leaf
153 from top of the plant and restricted by a nylon-mesh bag. One third-instar larva *S. exigua*, weighing
154 $14.6\pm 0.2\text{ mg}$ was infested on the first fully-developed leaf and also secured in a nylon-mesh bag.

155 Ten days after the infestation, all the beet armyworm larvae and aphids were removed from plants,
156 and then weight of caterpillars and the aphid individual number on each plant were measured. At the
157 same time, half the plants infested with nematodes and powdery mildew fungi were also sampled to
158 measure the galled roots and symptoms, fungal lesion area, of fungal infestation on tomato plants. In
159 addition, on the 20th day after pest infestation, the number of nematode galls and the fungal lesion area
160 were as well measured on the remaining number of plants.

161

162 **Data analyses**

163 All the data were analyzed by Kolmogorov-Smirnov test and were normal. Aphid population, weight
164 gains of beet armyworm larvae, fungal lesion area and nematode galls were analyzed with the univariate
165 analysis in a linear model followed by a Tukey's HSD test for multiple comparisons to assess the effects
166 of presence or absence of the other three species on one organism development. The correlation between
167 pest performance and the abundance of indirect interactions was analyzed using the Pearson correlation.
168 All analyses were performed with SPSS 25.0 (SPSS Inc, Chicago, USA).

169

170 **Results**

171 *Plant-mediated indirect interactions between two pests*

172

173 When two different functional types of pests attacked the tomato plant, there was no significant
174 difference in the number of nematode galled roots between plants with two different pest infestation and
175 the plants inoculated by nematodes alone on the 10th day post-infestation ($F_{3,16} = 0.840$; $P = 0.492$;
176 Fig.1a). However, on day 20 post-infestation, aphids and powdery mildew significantly facilitated
177 infection of nematodes on tomato roots ($F_{3,16} = 16.505$; $P < 0.001$; Fig.1a). Furthermore, aphids had
178 positive effects on the reproduction of powdery mildew on tomato leaves ($F_{3,16} = 7.422$; $P = 0.002$;
179 Fig.1b). Beet armyworms and powdery mildews significantly suppressed aphid abundance on tomato
180 plants ($F_{3,36} = 14.801$; $P < 0.001$; Fig.1c). Nevertheless, no effect was observed on the weight gains of
181 beet armyworm larvae when it shared the host with aphids, powdery mildew or nematodes ($F_{3,35} = 1.311$;
182 $P = 0.286$; Fig.1d).

183

184 *Plant-mediated indirect interactions among three pests*

185

186 When three different plant attackers simultaneously arrived on the tomato plant, the number of nematode
187 galls was significantly reduced in the plant jointly infested with aphids and powdery mildew fungi,
188 compared to that on the host attacked only by nematodes on day 10 post-infestation ($F_{3,16} = 10.142$; $P =$
189 0.001 ; Fig.2a), but the difference in the quantity of nematode galls vanished on day 20 post-infestation
190 ($F_{3,16} = 0.824$; $P = 0.500$; Fig.2a). There was no fungal symptom difference on tomato leaves between
191 the plants undergoing simultaneous attacks by three different organisms and those solely inoculated with
192 powdery mildew fungi. Nonetheless, fungal reproduction was remarkably enhanced on the tomato plants
193 jointly infested with beet armyworms and aphids compared to other three-pest-attacking plants ($F_{3,16} =$
194 3.898 ; $P = 0.029$; Fig.2b). In addition, the number of aphid individuals was significantly lower on the
195 plants simultaneously challenged by beet armyworms and nematodes and the plants attacked jointly by
196 powdery mildew fungi and nematodes as opposed to that on the plants only infested with aphids ($F_{3,36} =$
197 10.615 ; $P < 0.001$; Fig.2c). The development of beet armyworm larvae significantly accelerated on the
198 plants subjected to the combined infestation with aphids and powdery mildew fungi. Moreover, the
199 weight gains of beet armyworm larvae were significantly reduced on the plant corporately attacked with
200 powdery mildew and nematodes compared to the other three-pest-infested plants ($F_{3,35} = 6.954$; $P =$
201 0.001 ; Fig.2d).

202

203 *Plant-mediated indirect interactions among four pests*

204

205 When the tomato plant was simultaneously attacked by 4 different pests, the number of galled roots did
206 not significantly differ from that on plants only infested by nematodes. This was not only observed on
207 the 10th day ($F_{1,8} = 0.448$; $P = 0.522$; Fig.3) but also on the 20th day after nematode invasion ($F_{1,8} = 0.395$;
208 $P = 0.547$; Fig.3a). Furthermore, there was also no distinction on the reproduction of powdery mildew
209 ($F_{1,8} = 0.284$; $P = 0.608$; Fig.3b) and aphid population ($F_{1,18} = 0.005$; $P = 0.943$; Fig.3c) between the
210 plants which were challenged by 4 organisms and the plants attacked by aphids or powdery mildew alone.
211 However, the development of beet armyworm larvae was significantly bettered when 4 different
212 organisms shared the same host ($F_{1,18} = 6.880$; $P = 0.017$; Fig.3d).

213

214 *Correlation between pest performance and the indirect interaction abundance*

215

216 When different pests attacked tomato plants at the same time, both the number of aphid individuals ($r =$
217 0.286 ; $P = 0.017$; Fig.4a) and the weight gains of beet armyworm larvae ($r = 0.277$; $P = 0.022$; Fig.4b)
218 were positively correlated to the abundance of indirect interactions. However, there was no correlation
219 between indirect interaction abundance and fungal reproduction ($r = -0.191$; $P = 0.271$) as well as the
220 number of nematode galls either on the 10th day ($r = -0.050$; $P = 0.775$) or on the 20th day ($r = -0.107$; P
221 $= 0.540$) after nematode invasion.

222

223 **Discussion**

224 In the agroecosystem, plants are often simultaneously affected by different types of organisms both
225 aboveground and belowground. Indeed, the more phytophagous species are compatible with plants, the
226 more complex the mechanisms involved and the more diverse outcomes of indirect interactions may

227 occur. Therefore, it would be helpful to predict the pest population dynamics involved in the indirect
228 interactions among multiple attackers on plants, if a general pattern was obtained between pest
229 performance and pest biodiversity or the abundance of such indirect interactions. Our results indicated
230 that insect pests, aphids and beet armyworms, benefited from the increase of interaction abundance
231 whereas no correlation was found between the performance of pathogenic organisms, i.e. fungi and
232 nematodes, and the indirect interaction abundance when pests simultaneously attacked the tomato plant.
233 In addition, when two different feeding guilds of pests attacked the tomato plant at the same time, the
234 above-belowground indirect interactions between pests are unidirectional. However, bidirectional effects
235 between aboveground pests and belowground nematodes were observed when three different feeding
236 strategies of pests simultaneously consumed the host plant. Furthermore, beet armyworms were solely
237 successful in the indirect interactions among these four different feeding guilds of plant attackers on one
238 tomato plant.

239 When two different feeding guilds of pests attacked tomato plants, the aboveground pests, i.e., beet
240 armyworms and powdery mildew fungi, have stronger inhibitory influence on aphid population
241 compared to belowground nematodes. It may be due to both the synergism of the same SA hormone
242 signaling pathways induced by pathogens as well as aphids, and the reallocation of resource from leaves
243 to roots under defoliation by caterpillars (Erb et al., 2009; Soler et al., 2012a; Thaler et al., 2012; Ueda
244 et al., 2019). According to the optimal defense theory that defenses are costly to plants and should be
245 preferentially allotted to parts under the greatest risk. Therefore, it admits that foliar consumers elicit
246 elevated resistance in aboveground part compared to the little to no alterations of defensive phenotype
247 in roots (Kaplan et al., 2008; Eisenring et al., 2017). However, the mutual confrontation (Moultet et al.,
248 2011) is not always observed between aphids and fungi. Our results demonstrated that aphid population

249 was suppressed by fungal infestation but fungal reproduction was boosted by aphids. Besides
250 phytohormonal synergy, the asymmetrical interactions between these two organisms may also rely on
251 modulation of source-sink tissue relationship. To this effect, plant pathogens turn tissues from sources
252 into sink, leading to resource conservation in local tissue and hence negative influences on carbohydrate
253 supply in systemic tissue for aphid feeding (Lemoine et al., 2013). Furthermore, co-infestation with
254 aphids or fungi, significantly enhanced root infection by nematodes, while combined attack by
255 caterpillars on tomato plants decreased infection rate of nematodes. These results were also supported by
256 the hypothesis of interactions between phytohormone signaling pathways, namely, the JA signaling
257 pathway induced by nematodes in roots was interfered with aphid-mediated or pathogen-mediated SA
258 signaling, yet synergized with the same type of JA signaling triggered by caterpillars on tomato leaves
259 (Soler et al., 2012a; Coppola et al., 2013). However, it seems that aphids or powdery mildew fungi, which
260 cause slight mechanical wounds on hosts may be more susceptible to aboveground organisms compared
261 to the beet armyworm as a chewing insect causing leaf defoliation. The performance of caterpillars was
262 not altered by other organisms when two pests simultaneously attacked tomato plants, which is consistent
263 with the investigation carried out by Eisenring et al (2018). That neither aphid nor whitefly infestation
264 impacted caterpillar development may be due to the lack of systemic induction of secondary metabolites
265 by phloem-feeding herbivores (Eisenring et al., 2018). Our results demonstrated that pests feeding on
266 shoots significantly impacted the performance of both aboveground and belowground phytophages when
267 only two different pests simultaneously attacked tomato plants, rather than vice versa. Indeed,
268 performance of aboveground attackers was more easily altered by co-occurrence of other aboveground
269 consumers compared to the only belowground challenger (nematodes in this study). This may be likely
270 linked to the stronger and faster systemic defenses within the same part of plant, the shorter distance to

271 transport defensive compounds, and a lower defense cost. The asymmetrical impact of aboveground-
272 belowground interactions on shoot and root phytophages may rely on the organ specificity of defensive
273 induction. It has been reported that different organs, e.g. the roots and shoots, or the leaves and fruits,
274 are present distinctively in specific sets of defensive genes or secondary metabolites induced by
275 aboveground phytophages (Biere & Goverse, 2016). For example, leaf inoculation with fungal pathogen,
276 *Colletotrichum graminicola*, significantly elevated both SA levels and expression of PR-related genes
277 on maize shoots but only the oxylipin-related genes were upregulated in roots (Balmer et al., 2013). In
278 addition, expression of both JA and ET biosynthesis genes was enhanced in the aboveground part of the
279 maize through defoliation by *S. frugiperda* on leaves, while only increased ET signaling was detected in
280 roots (Ankala et al., 2013). Furthermore, organ-specific immune responses also play an important role in
281 shaping the outcome of aboveground-belowground interactions, on account of substantial differences
282 between defense responses initiated by shoots and those initiated by roots, indicating the significance of
283 the location of inducer and responder (Balmer & Mauch-mani, 2013). For instance, expression of
284 defense-related genes was distinctly upregulated in rice shoots along with infection by *Magnaporthe*
285 *oryzae* on rice shoots, while it was suppressed in roots after the attack by *M. oryzae* on roots (Marcel et
286 al., 2010).

287 When three species of consumers fed on the host, aphid population was significantly suppressed by
288 the co-presence of the belowground nematodes, likely possessing the similar mechanism with the shoot-
289 to-root-to-shoot defensive loop. The defensive genes in roots could be triggered to activate the synthesis
290 of defense compounds after an aboveground organism attack, thereby contributing to the aboveground
291 defense against aphids (Orlowska et al., 2012; Louis & Shah, 2013; Fragoso et al., 2014). Moreover,
292 Addition of the nematode-induced defenses strengthened the defenses from belowground to aboveground

293 in this loop (Guo & Ge, 2017). It is consistent with our results as shown in Fig 2, when three different
294 pests attacked the host, participation of belowground nematodes led to a greater inhibition on
295 performance of all the aboveground organisms, compared to interactions within aboveground pests. In
296 addition, when three aboveground pests attacked the host, facilitation to the development of caterpillar
297 larvae, once again supported the hypothesis that JA signaling induced by caterpillars maybe strongly
298 suppressed by both the aphid-induced and pathogen-induced SA signaling. Therefore, the production of
299 repellent terpenes dependent on JA signaling was reduced, leading to an acceleration of feeding by *S.*
300 *exigua* on tomato leaves (Bosch et al., 2014). Intriguingly, strongly antagonistic interactions were
301 observed among nematodes, powdery mildew fungi and aphids resulting in slightly decreased fungal
302 reproduction, significantly inhibited aphid population and distinctly attenuated nematode infection rate
303 on roots. Thus, this could explain why only the caterpillar benefitted from the simultaneous attack by all
304 four plant consumers on tomato plants.

305 Facilitating insect performance with the increasing interaction abundance may be the result of a
306 trade-off by the host between pest-induced defense and maximization of their fitness. Generally, as
307 reported in compatible plant-herbivore interactions, the activation of induced defense was accompanied
308 by downregulation of photosynthesis genes (Bilgin et al., 2010; Coppola et al., 2013). In addition, the
309 simultaneous activation of multiple different defensive pathways or defensive mechanisms caused by the
310 various pest attack on the host may as well attenuate the asymmetry of interactions among different pest-
311 induced defenses (Erb et al., 2011). In order to balance the cost between plant defenses and their fitness,
312 the magnitude of plant defenses assigned against a certain species of pest would gradually decrease with
313 the more abundant interactions because in which case the host needs greater cost to defend against more
314 biotypes. Nevertheless, on the basis of our results, it seems that the defense-fitness balance hypothesis

315 works better on insects than pathogen biotypes. On the one hand, it may be connected to the different
316 immune systems between insect pests and pathogenic organisms that respond to plant induced defenses.
317 On the other hand, more diverse interactions involving more species of phytophagous biotypes should
318 be evaluated to verify such a hypothesis.

319 Overall, in this study, a general relationship was obtained that herbivorous insects benefitted from
320 the increase of indirect interaction abundance when multiple functional types of pests attacked the tomato
321 at the same time. In addition, the direction between aboveground-belowground pest interactions as well
322 altered with the more abundant indirect interactions. Our results support that the plant-mediated indirect
323 interactions between two pests may largely depend on the interactions of phytohormonal signaling
324 pathways but such a hypothesis should be further verified in the next study via analyzing the host
325 metabolomics. However, with the increase of pest species involved in plant-mediated indirect
326 interactions, the integrated defensive loops and host resource dynamics changes may play the increasing
327 role in these indirect interactions due to their tighter interconnections in the host. Finally, in addition to
328 focusing on the phytophagous performances and host metabolomics, the pest population dynamics
329 monitoring and the attractiveness to a higher trophic level, such as parasitoids, would be our next
330 objectives to study the long-term effects of such indirect interactions in practice. Consequently, our study
331 may act as a reference to predict the outcome of indirect interactions connecting aboveground and
332 belowground pests with different biodiversity, and provide suggestions or backup to further contextualize
333 plant integrated defense strategies against diverse biotic stresses, as well as favor integrated pest
334 management in the agroecosystem.

335

336 **Acknowledgements**

337 We would like to thank Nathalie Marteu, Jean-Francois Bourgeay and Dr. Salvador Herrero for providing
338 the nematodes, powdery mildews and beet armyworms used in this study. This work was funded by the
339 European Union's Horizon 2020 research and innovation program (Grant number: 633999) and China
340 Scholarship Council.

341

342 **Author contributions**

343 YQ, AVL and ND conceived and designed the research. PCS and PCN provided comments to the study.
344 YQ and PB performed experiments. YQ, LSM and ND carried out data analysis. YQ, AVL, LSM, PCS
345 and ND wrote the manuscript. PCS, PCN and ND contributed materials and methods. All authors read
346 and approved the manuscript.

347

348 **Compliance with ethical standards**

349 **Conflict of interest** All authors declared that they have no conflict of interest.

350 **Ethical approval** This article does not contain any studies that required human or animal approval.

351

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488 **Table legends**

489 **Table 1** Treatments with different pest infestation on tomato plants in the study

| Treatment | 1 pest | | | | 2 pests | | | | 3 pests | | | | 4 pests | | | |
|--------------------------|--------|---|---|---|---------|---|---|---|---------|---|---|---|---------|---|---|---|
| <i>S. exigua</i> | + | - | - | - | + | + | + | - | - | - | - | + | + | + | - | + |
| <i>M. euphorbiae</i> | - | + | - | - | + | - | - | + | + | - | + | + | + | - | + | + |
| <i>O. neolycopersici</i> | - | - | + | - | - | + | - | + | - | + | + | + | - | + | + | + |
| <i>M. incognita</i> | - | - | - | + | - | - | + | - | + | + | + | - | + | + | + | + |

490 + means that the pest was presence in the treatment; - means absence of the pest in the treatment.

491 **Figure legends**

492 **Fig.1** Effects on the life-history traits of nematodes (a), powdery mildews (b), aphids (c) and beet
493 armyworms (d) when tomato plants were simultaneously attacked by 2 pests.

494 Me: *M. euphorbiae*; On: *O. neolycopersici*; Mi: *M. incognita*; Se: *S. exigua*; the black bars and grey bars
495 indicated pest performance on day 10 and day 20 post-infestation, respectively; results of fungal
496 reproduction on day 10 post-infestation were not shown as no obvious fungal symptom was observed on
497 leaves; capital letters in (a) indicates the comparison among different treatments in group day 10;
498 different letters in each group indicates the significant difference at $P < 0.05$.

499

500 **Fig.2** Effects on the life-history traits of nematodes (a), powdery mildews (b), aphids (c) and beet
501 armyworms (d) when tomato plants were simultaneously attacked by 3 pests.

502 Me: *M. euphorbiae*; On: *O. neolycopersici*; Mi: *M. incognita*; Se: *S. exigua*; the black bars and grey bars
503 indicated pest performance on day 10 and day 20 post-infestation, respectively; results of fungal
504 reproduction on day 10 post-infestation were not shown as no obvious fungal symptom was observed on
505 leaves; capital letters in (a) indicates the comparison among different treatments in group day 10;
506 different letters in each group indicates the significant difference at $P < 0.05$.

507

508 **Fig.3** Effects on the life-history traits of nematodes (a), powdery mildews (b), aphids (c) and beet
509 armyworms (d) when tomato plants were simultaneously attacked by 4 pests.

510 Me: *M. euphorbiae*; On: *O. neolycopersici*; Mi: *M. incognita*; Se: *S. exigua*; the black bars and grey bars
511 indicated pest performance on day 10 and day 20 post-infestation, respectively; results of fungal
512 reproduction on day 10 post-infestation were not shown as no obvious fungal symptom was observed on

513 leaves; capital letters in (a) indicates the comparison among different treatments in group day 10;

514 different letters in each group indicates the significant difference at $P < 0.05$.

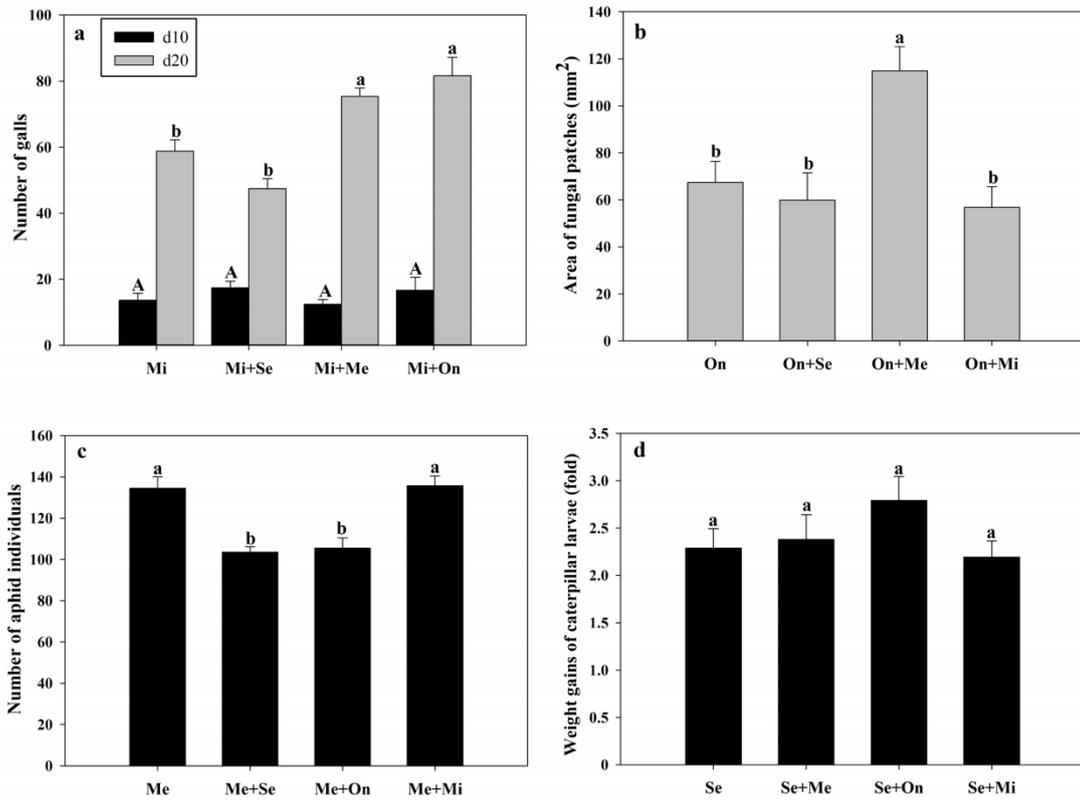
515

516 **Fig.4** Relationship between life-history traits of aphids (a) and beet armyworms (b) and the abundance

517 of indirect interactions when tomato plants were simultaneously attacked by different pest species.

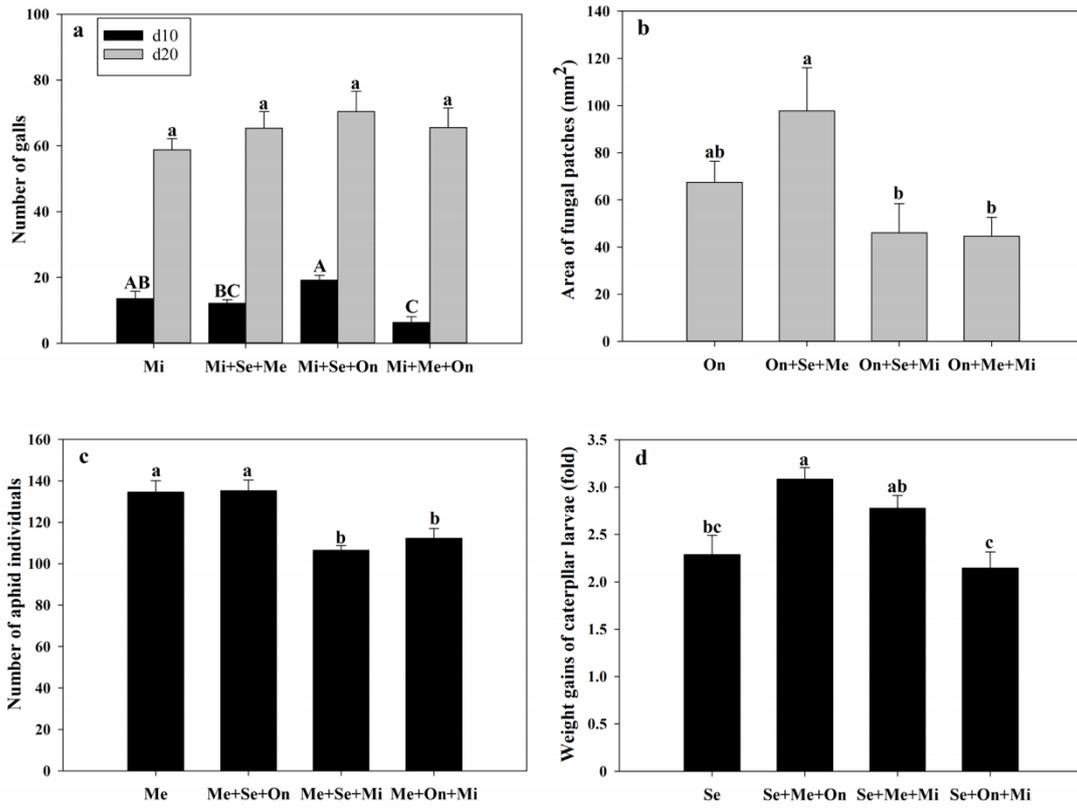
518

519



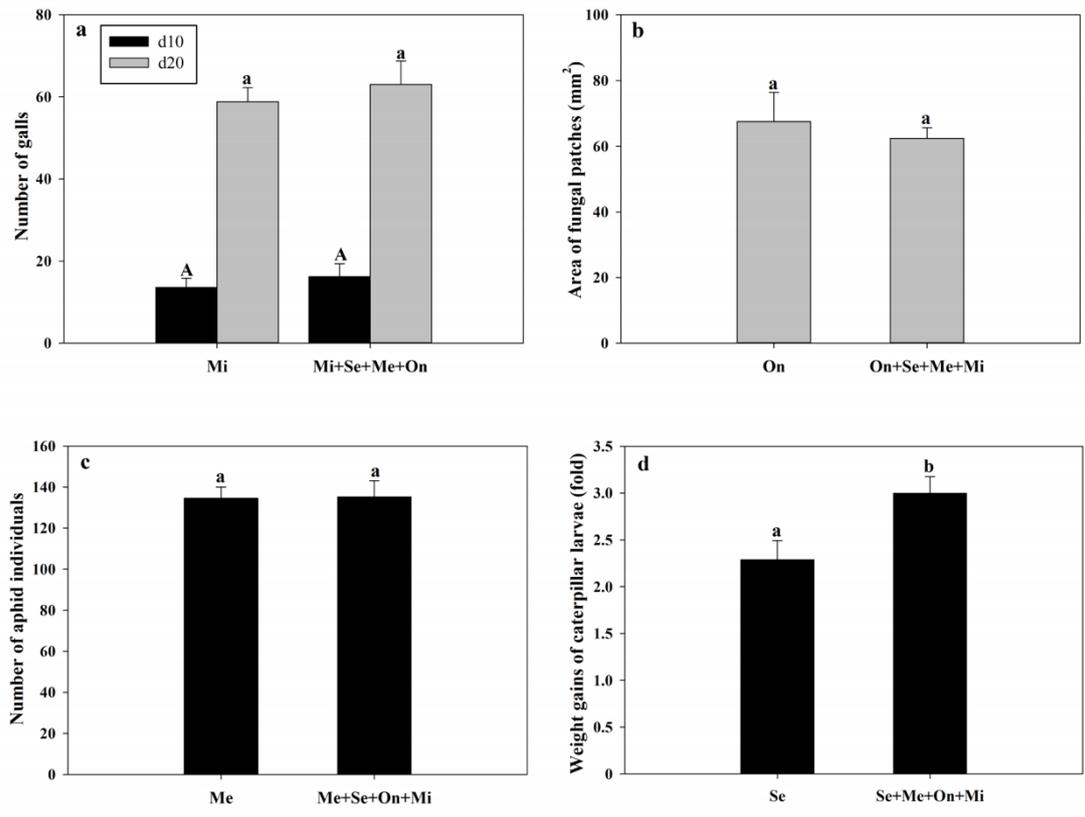
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521 **Figure 1**



522

523 **Figure 2**



524

525 **Figure 3**

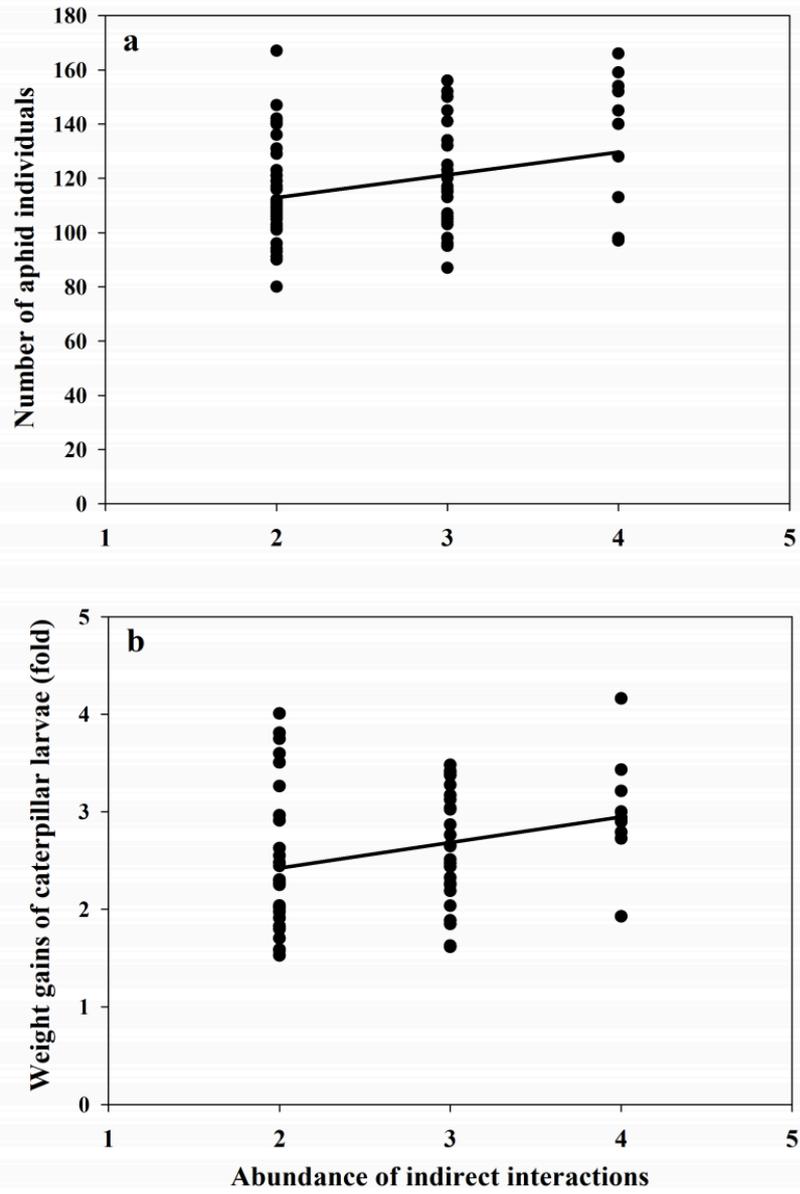


Figure 4

2. Impact of biodiversity on plant-mediated indirect interactions under sequential pest infestation

Article 2

Qu YY, Lavoit AV, Amiens-Desneux E, Monticelli LS, Wang YS, Castagnone-Sereno P, Nicot PC, Desneux N

Impact of biodiversity on plant-mediated indirect interactions under sequential pest infestation.

In preparation

1 **Impact of biodiversity on plant-mediated indirect interactions under sequential**
2 **pest infestation.**

3

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10

11

12

13

14 **Abstract**

15 Plants can be consumed by a plethora of organisms, mainly including herbivorous
16 insects and plant pathogenic organisms, such as viruses, bacteria, fungi and nematodes.
17 In this study, four different feeding strategies of pests, i.e. the piercing-sucking aphid
18 (*Macrosiphum euphorbiae*), the chewing caterpillar (*Helicoverpa armigera*), the plant
19 fungal pathogen (*Oidium neolycopersici*) and the root-knot nematode (*Meloidogyne*
20 *incognita*) were sequentially introduced on tomato plant, *Solanum lycopersicum* L, to
21 evaluate the plant-mediated indirect interactions among these pests. The assessment of
22 such the indirect interactions was conducted with different number of pest species. The
23 unidirectional interaction between aboveground and belowground pests was found in
24 the current study. Performance of pests that attacked tomato shoots was significantly
25 affected (both positively and negatively) by the co-exist of belowground nematodes.
26 Pests early arrived on tomatoes had influences on the performance of pests that
27 subsequently attacked the same host. What's more, the negative correlation was
28 detected between the insect performance, i.e. the number of aphid individuals and the
29 development rate of cotton bollworm larvae, and the abundance of indirect interactions.
30 However, such a relationship was not found between the performance of pathogenic
31 organisms, i.e. fungal reproduction and inoculating rate of nematodes, and the number
32 of pest species involved in the indirect interactions. Furthermore, the positive
33 correlation was observed between plant growth, i.e. the growth of plant height and stem
34 nodes, and the species number involved in such indirect interactions. Our results may
35 lead to formalization of plant-mediated indirect interactions among multiple pests in an

- 36 agroecosystem model in favor of the development of more efficient Integrated Pest
37 Management programs.
- 38 **Key words:** plant-mediated indirect interactions; above-below ground interactions;
39 feeding guilds; biodiversity; insect performance; plant fitness

40 **Introduction**

41 Plants are often attacked by a diverse community of phytophagous enemies, including
42 insects and pathogenic organisms. Upon attack by these biotic stresses, plants can
43 employ both constitutive and induced defense against the bioaggressors (Wittstock and
44 Gershenzon, 2002; Dicke et al., 2003; Kaplan et al., 2008, Hatt et al., 2019).

45 Such defenses resulted in an array of physiological or chemical changes in plants, and
46 thereafter have an influence on the subsequent attackers on the same host (Gatehouse,
47 2002; Mouttet et al., 2011). The plant-mediated indirect interactions would affect both
48 plants, pests, and even a third trophic levels, and could cause negative, positive, or
49 neutral impact on pests (Gatehouse, 2002; Mouttet et al., 2011, 2013; Poelman and
50 Dicke, 2014). For instance, when monarch caterpillars *Danaus plexippus* and oleander
51 aphids *Aphis nerii* shared the same host milkweeds, aphid population was significant
52 reduced by caterpillars, but the caterpillar development benefited from such indirect
53 interactions (Ali and Agrawal, 2014). Plant defenses induced by the early arriving
54 attackers may not only affect the behavior, survival, development and fecundity of
55 subsequently arrivals but also have influences on the population dynamics (Poelman et
56 al., 2008).

57 Such plant-mediated indirect interactions could be modulated by various biotic
58 and abiotic factors, including pest attacking sequence, attacking location, pest feeding
59 strategy, host specialization, water, salt and nutrient (Ali and Agrawal, 2014; Barber et
60 al., 2015; Kroes et al., 2016; Gaillard et al., 2018). Induced plant defensive compounds
61 accumulated not only at the local feeding site, but also in undamaged tissues (Mouttet

62 et al., 2011, 2013). Hence, the plant-mediated interactions caused by systemic
63 responses are spatiotemporal-dependent (Moultet et al., 2011, 2013). The order of pests
64 attacking the plant can greatly impact the performance of other herbivores that arrived
65 later or earlier on the same plant (Erb et al., 2011). When aphids *Brevicoryne brassicae*
66 and caterpillars *Pieris brassicae* attacked *Brassica oleracea* together, caterpillar
67 performance benefited more from the simultaneous attack than sequential attack by the
68 two species of herbivores (Soler et al., 2012a). Moreover, such indirect interaction
69 between plant attackers are not restricted to the aboveground part of plants, and they
70 can link shoots and roots parts via systemically induced plant defenses (Soler et al.,
71 2012b, 2013; Lee et al., 2017; Kundu et al., 2018). Attack by wireworms *Agriotes*
72 *lineatus* on cotton roots significantly increased the terpenoid defensive substances in
73 cotton leaves, and thereby largely reduced leaf consumption by the aboveground
74 chewing insect *Spodoptera exigua* (Bezemer et al., 2003).

75 Different feeding guilds of plant challengers often triggered different
76 phytohormone signaling pathways, which mainly mediated by plant hormones such as
77 jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) (Stout et al. 2006; Di et al.
78 2017). Distinct defensive signaling pathways may interact directly and indirectly to
79 form a complex signaling network in plants, and these interactions may be additive,
80 antagonistic or synergistic (Koornneef and Pieterse, 2008; Leon-Reyes et al., 2010).
81 Interactions between the hormone pathway triggered by the attackers would shape the
82 outcome of plant-mediated indirect interactions among pests which share the same host
83 (Stout et al., 2006).

84 In this current study, four different feeding strategies of pests was used to assess
85 the plant-mediated indirect interactions among them. The indirect interaction would be
86 examined under distinct pest biodiversity to explore whether a general relation exists
87 between the pest performance and the number of pest species sharing the same host.
88 Moreover, the plant growth and development including plant height and stem nodes
89 was also considered to evaluated the impact of pest biodiversity involved in such an
90 indirect interaction on the plant fitness. It may serve as a reference for the study on the
91 indirect above-below ground interactions among multiple pests and also show a
92 suggestion to predict the development trends of plant disease and insect pests in
93 agroecosystem.

94

95 **Materials and methods**

96

97 *Study organisms*

98

99 Tomato plants, *Solanum lycopersicum*, Solanaceae (cultivar ‘San Marzano Nano’),
100 were grown in a climate controlled greenhouse ($23\pm 0.3^{\circ}\text{C}$ in daytime, $18\pm 0.2^{\circ}\text{C}$ at night,
101 RH: $66\pm 1\%$, irradiance: $663\pm 48 \text{ W/m}^2$). The seedlings were planted in the substrate
102 combination which contains soil and perlites and supplied with a standard nutrient
103 solution (pH=6, electrical conductivity (EC) = 1.6 mS/cm , ion concentration: 620 mg/L
104 NO_3^- , 11 mg/L NH_4^+ , $170 \text{ mg/L H}_2\text{PO}_4^-$, $115 \text{ mg/L SO}_4^{2-}$, 180 mg/L K^+ , 212 mg/L Ca^{2+} ,
105 12 mg/L Mg^{2+}).

106 The *M. incognita* nematode isolate used in this study was from the collection in
107 INRA, Sophia Antipolis, France. Infective second-stage juveniles (J2) were recovered
108 from infested tomato plants which grew under greenhouse conditions according to the
109 procedure of Neveu et al. (2003). The *O. neolycopersici* isolate came from tomato
110 plants in INRA, Avignon, France. Spores suspensions were prepared by adequately
111 blending the fully-infested tomato leaves with distilled water. The supernatant was
112 collected for the subsequent quantification of spore concentration which was measured
113 with a Mallasez[®] cell and adjusted to a final concentration of 10⁵ spores mL⁻¹. The *H.*
114 *armigera* colony was established from the individuals found by Mr. Marcel Caporalino
115 in the field (Sophia Antipolis). The cotton bollworm colony was maintained by
116 continuous supply of artificial food in climatic chambers at INRA (L: D = 16:8, 24±1 °C,
117 55±5% RH). The colony of aphids, *M. euphorbiae*, was reared on the caged tomato
118 plants at INRA, Sophia Antipolis, France.

119

120 *Experimental design*

121

122 Fifteen different infestation treatments respectively including one, two, three or four
123 pests were conducted on tomato plants (Table 1). Plants which were not infested with
124 any pests was as the control. Each plant represented one replicate and each treatment
125 was replicated 9 times.

126 The four different pests were sequentially introduced onto tomato plants in the
127 climatic greenhouse (23±0.3°C in daytime, 18±0.2°C at night, RH: 66±1%, irradiance:

128 663±48 W/m²). At DAS 35 (Days After Seeding), plant height and the number of stem
129 are measured, and then the *M. incognita* inoculum containing 200 J2 was inoculated
130 into the soil around tomato roots. At DAS 44 the *O. neolycopersici* suspension of 10
131 µL was introduced onto one leaflet (the third fully-developed leaf from the top of the
132 tomato plant) and it was isolated in a Petri dish (diameter = 10cm, height = 7cm). A
133 3cm diameter hole was dug on the cap and covered with nylon mesh, which facilitates
134 moisture reduction inside the dishes. Twenty third-instar aphids were settled on the leaf
135 which is one above the fungi-infestation leaf and restricted by a nylon-mesh bag at DAS
136 51. At DAS 55 one second-instar *H. armigera* larva after weighting was infested on the
137 leaf which is most adjacent above the aphid-infestation leaf. The *H. armigera* larva was
138 as well secured in a nylon-mesh bag. All the pest treatments and control were measured
139 at DAS 61, i.e. the number of nematode galls and aphid individuals were counted; the
140 fungal lesion area was measured; the weight of cotton bollworm larva was recorded;
141 plant height and the number of stem nodes in all the treatments were as well measured.

142

143 **Data analyses**

144

145 All data were analyzed using R version 3.4.0. To analyze the effects of the presence or
146 absence of three other species on a pest development, a linear model was used. The
147 normality of the data was previously tested and when it was necessary, a square-root
148 transformation was performed to achieve the normality data. The impact of pest
149 presence on the increase of plant height was also analyzed by a linear model and the

150 increasing number of node per plant was analyzed by general linear model with Poisson
151 distribution. The residues of linear model were tested and they were normal. Multiple
152 comparison of mean values was done with the 95 % confidence level using the
153 ‘multcomp’ package. The correlation between the abundance of indirect interactions
154 among pests and the performance of each pest as well as the plant fitness was analyzed
155 with a Pearson correlation.

156

157 **Results**

158

159 *Plant-mediated indirect interactions between two pests*

160

161 When two different pests attacked tomato plants sequentially, only the weight gains of
162 cotton bollworm larvae were increased by nematodes ($F_{3,22} = 4.979$; $P = 0.009$; Fig.1d).

163 The performance of other three pests, i.e. then number of aphid individuals ($F_{3,29} =$
164 1.374 ; $P = 0.270$; Fig.1c), the number of nematode galls ($F_{3,32} = 0.750$; $P = 0.530$;
165 Fig.1a) and the area of fungal patches ($F_{3,32} = 0.480$; $P = 0.696$; Fig.1b), were not
166 affected by the other pest which shared the same host.

167

168 *Plant-mediated indirect interactions between three pests*

169

170 When three pests consumed the host at different time, the area of fungal patches on
171 tomato plants jointly attacked by aphid and nematodes was significantly larger than that

172 on plants only infested by powdery mildew fungi ($F_{3,32} = 3.980$; $P = 0.016$; Fig.2b).
173 However, the individual number of aphids on the tomato plants together challenged by
174 powdery mildew fungi and nematode distinctly decreased, compared to the plants
175 solely infested by aphids ($F_{3,30} = 4.075$; $P = 0.015$; Fig.2c). No significant difference
176 was detected on the number of nematode galls ($F_{3,30} = 0.580$; $P = 0.630$; Fig.2a) and
177 weight gains of cotton bollworm larvae ($F_{3,21} = 1.399$; $P = 0.271$; Fig.2d) between the
178 tomato plants consumed by three pests and the plants only attacked by nematodes or
179 caterpillars.

180

181 *Plant-mediated indirect interactions between four pests*

182

183 When four different functional types of pests sequentially attacked tomato plants, the
184 number of aphid individuals was significantly reduced ($F_{1,14} = 10.730$; $P = 0.006$;
185 Fig.3c). Nevertheless, there was no remarkable impact on the nematode galled roots
186 ($F_{1,15} = 2.600$; $P = 0.130$; Fig.3a), area of fungal patches ($F_{1,16} = 0.001$; $P = 0.970$;
187 Fig.3b) and weight gains of cotton bollworm larvae ($F_{1,13} = 2.105$; $P = 0.171$; Fig.3d).

188

189 *Correlation between pest performance and the indirect interaction abundance*

190

191 When different pests attacked tomato plants in sequence, both the number of aphid
192 individuals ($r = -0.257$; $P = 0.043$; Fig.4a) and the weight gains of cotton bollworm
193 larvae ($r = -0.335$; $P = 0.028$; Fig.4b) were negatively correlated to the number of pest

194 species involved in the indirect interactions. However, there was no correlation between
195 the abundance of indirect interaction and fungal reproduction ($r = 0.108$; $P = 0.401$) as
196 well as the number of nematode galls ($r = -0.002$; $P = 0.989$).

197

198 *Impact of plant-mediated indirect interactions on plant fitness*

199

200 There was no difference in the growth of plant height ($F_{4,40} = 0.974$; $P = 0.433$; Fig.5a)
201 and plant nodes ($F_{4,40} = 0.595$; $P = 0.668$; Fig.5b) between control plants and one-pest-
202 infested plants. Nevertheless, plant growth both in height ($r = 0.219$; $P = 0.029$; Fig.6a)
203 and in stem nodes ($r = 0.229$; $P = 0.023$; Fig.6b) were positively correlated with the
204 abundance of indirect interactions among pests when they attacked tomato plants in
205 sequence.

206

207 **Discussion**

208 In our study, the unidirectional interaction between belowground and aboveground
209 pests was detected. Pests attacking shoots of tomato plants were significantly affected
210 by plant-mediated indirect interactions, while there were no influences on the
211 performance of belowground nematodes. In addition, attacking sequence as well play
212 an important role in shaping the outcome of the indirect interactions mediated by the
213 host plant among multiple pests. Pests that arrived later among the indirect interactions
214 were easier to be impacted, compared to the first attacking pest. Moreover, the
215 performance of herbivorous insect (aphid and cotton bollworm) was significantly

216 negative correlated to the abundance of indirect interactions, and the alteration of plant
217 fitness (plant height and nodes) was remarkably positive correlated to the abundance of
218 indirect interactions.

219 Induced responses in plants may be the major determinants of ecological
220 interactions, especially on the latter arriving attackers. Pests that firstly arrived on the
221 plant could occupy the pre-option to manipulate the host. Therefore, the aphid, powdery
222 mildew and cotton bollworm, as the Johnny-come-lately, were susceptible in the
223 indirect interaction with the root-knot nematode that was the first attacker on the host.
224 Moreover, the interactions between signaling pathways depending on phytohormones,
225 such as JA and SA, appear to play important roles in determining community
226 composition (Koornneef and Pieterse, 2008). As reported by Guo and Ge (2017), the
227 root-knot nematodes increased SA-dependent signaling pathway in tomato root and
228 leaves. However, the chewing caterpillar may be responsible for the JA-dependent
229 signaling pathway in plants (Biere and Goverse, 2016; Bosch et al., 2014). Hence, the
230 facilitation to larva development of cotton bollworm caused by nematode pre-
231 infestation in tomatos may benefit from the SA-JA signaling antagonism (Caarls et al.,
232 2015; Thaler et al., 2012).

233 Not only do the plant-mediated indirect interactions depend on the interplay
234 between hormone signaling induced by plant attackers, but the alteration in resources
235 and sink-resource relationship also play a vital role in the outcome of such indirect
236 interactions (Biere and Goverse, 2016). It has been reported that plant pathogens could
237 turn tissues from sources into sink, leading to resource conservation in local tissue and

238 hence negatively affecting carbohydrate supply in systemic tissue for aphid feeding
239 (Lemoine et al., 2013). This may be linked to the asymmetrical effects on aphids (Fig.
240 2c) and mildew fungi (Fig. 2d), when nematodes, fungi and aphids attacked the same
241 host together. Furthermore, Guo and Ge (2017) also observed the decreased
242 concentration of nitrogen in tomato leaves after nematode inoculation. The decreased
243 nitrogen concentration may result in reduction of free amino acid in phloem, and
244 thereby negatively impact the aphid performance.

245 All the defensive plant traits altered by its attackers could shape the outcomes of
246 the indirect interactions among these attackers and construct the composition of
247 consumer community associated with the plant (Stam et al., 2014). Defensive
248 compounds produced by induced defenses may gradually accumulate with the increase
249 of successively attacking pests (Mewis et al., 2006). Hence, the pest exposed to more
250 abundant indirect interactions should suffer more by the multiple induced defenses of
251 plants (Eyles et al., 2010; Karban, 2011). In turn, the diversity of community or the
252 abundance of indirect interactions as well feeds back to plant fitness. Plants has a
253 tolerant capacity to maintain their fitness under the biotic stresses through the
254 reallocation of resources away from the attacking sites. Therefore, the growth
255 acceleration in the plant height and stem nodes caused by the more complex interactions
256 among multiple pest species could be a compensation of plant fitness as a response to
257 multiple pest attack (McNaughton, 1983; Boege, 2005). The tolerance comes from
258 those traits that do not primarily serve to defend against attackers, but to compensate

259 for damage through alterations in assimilation rate, compensatory growth, resource
260 allocation or morphological changes (Mitchell et al., 2016).

261 Our results may help further understanding of the indirect interactions among pests
262 mediated by plants, and thereby contribute to sustainable crop protection. Furthermore,
263 more different functional types of pests, such as cell content-feeding, leaf-mining, gall-
264 forming and nectar-feeding herbivores, and more number of pest species should be
265 involved in tests to validate and generalize the relationship between pest performance
266 (and/ or plant fitness) and pest biodiversity.

267

268 **Acknowledgements**

269 We would like to thank Nathalie Marteu, Jean-Francois Bourgeay and Marcel
270 Caporalino for providing the nematodes, powdery mildews and cotton bollworms used
271 in this study. This work was funded by the European Union's Horizon 2020 research
272 and innovation program (Grant number: 633999) and China Scholarship Council.

273

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388 **Table legends**

389 **Table 1** Treatments with different pest infestation on tomato plants in the study

| | Control | 1 pest | | | 2 pests | | | 3 pests | | | 4 pests | | | | | |
|----|---------|--------|---|---|---------|---|---|---------|---|---|---------|---|---|---|---|---|
| Ha | - | + | - | - | - | + | + | + | - | - | - | + | + | + | - | + |
| Me | - | - | + | - | - | + | - | - | + | + | - | + | + | - | + | + |
| On | - | - | - | + | - | - | + | - | + | - | + | + | - | + | + | + |
| Mi | - | - | - | - | + | - | - | + | - | + | + | - | + | + | + | + |

390 + means that the pest was presence in the treatment; - means absence of the pest in the

391 treatment; Ha: *H. armigera*; Me: *M. euphorbiae*; On: *O. neolyopersici*; Mi: *M.*

392 *incognita*

393 **Figure legends**

394 **Fig.1** Effects on the life-history traits of nematodes (a), powdery mildews (b), aphids (c) and
395 cotton bollworms (d) when tomato plants were sequentially attacked by 2 pests.

396 Me: *M. euphorbiae*; On: *O. neolycopersici*; Mi: *M. incognita*; Ha: *H. armigera*; different
397 letters indicated the significant difference at $P < 0.05$.

398

399 **Fig.2** Effects on the life-history traits of nematodes (a), powdery mildews (b), aphids (c) and
400 cotton bollworms (d) when tomato plants were sequentially attacked by 3 pests.

401 Me: *M. euphorbiae*; On: *O. neolycopersici*; Mi: *M. incognita*; Ha: *H. armigera*; different
402 letters indicated the significant difference at $P < 0.05$.

403

404 **Fig.3** Effects on the life-history traits of nematodes (a), powdery mildews (b), aphids (c) and
405 cotton bollworms (d) when tomato plants were sequentially attacked by 4 pests.

406 Me: *M. euphorbiae*; On: *O. neolycopersici*; Mi: *M. incognita*; Ha: *H. armigera*; different
407 letters indicated the significant difference at $P < 0.05$.

408

409 **Fig.4** Correlation between life-history traits of aphids (a) and cotton bollworms (b) and the
410 abundance of indirect interactions when tomato plants were sequentially attacked by different
411 pest species.

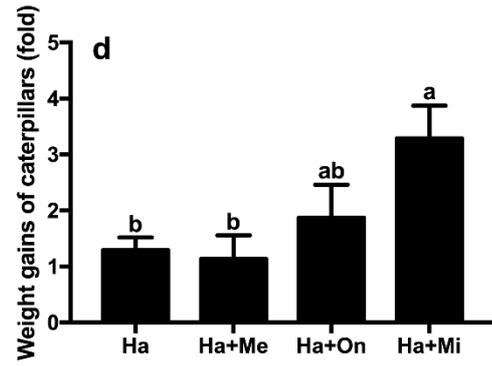
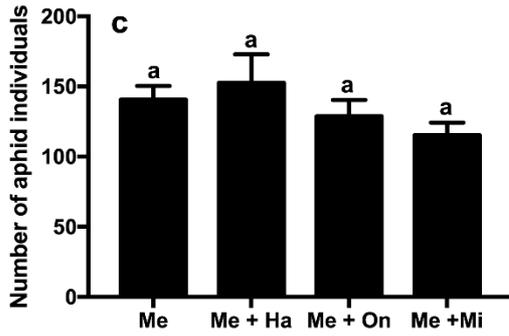
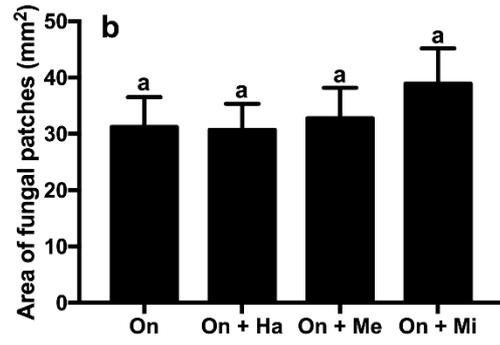
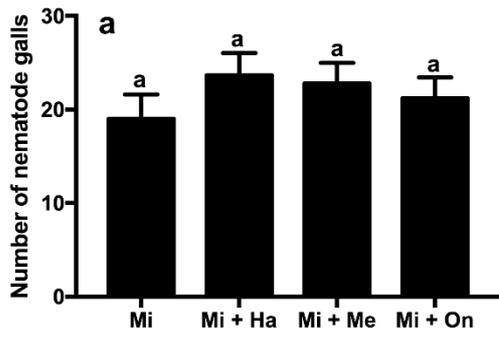
412

413 **Fig.5** Impact of one pest consumption on plant height growth (a) and the growth of plant
414 nodes (b).

415 Me: *M. euphorbiae*; On: *O. neolycopersici*; Mi: *M. incognita*; Ha: *H. armigera*

416

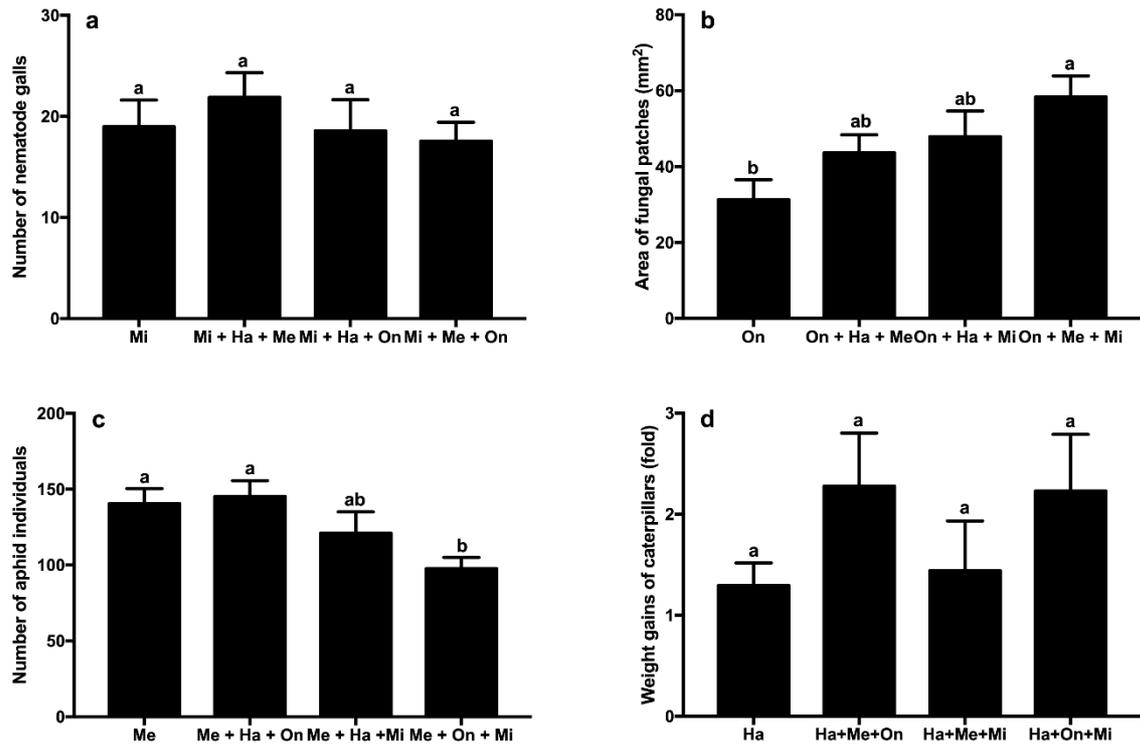
417 **Fig.6** Correlation between plant height growth (a) and the growth of plant nodes (b) and the
418 abundance of indirect interactions when tomato plants were sequentially attacked by different
419 pest species.



420

421 **Figure 1**

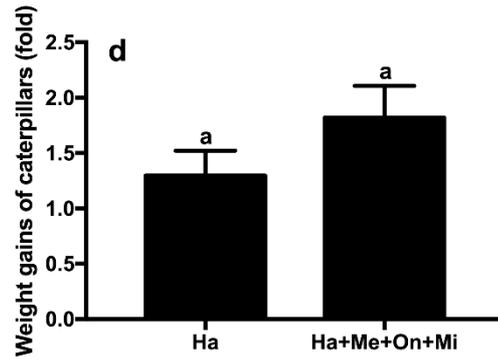
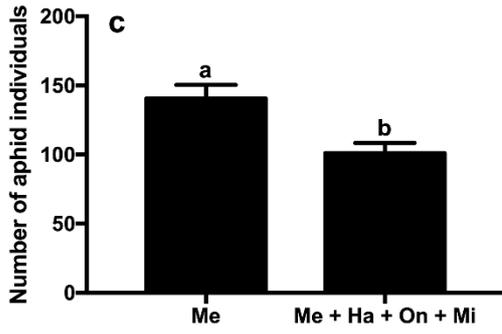
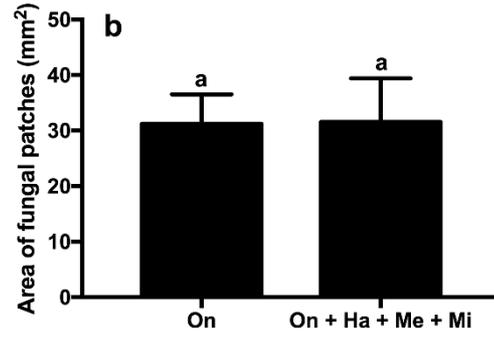
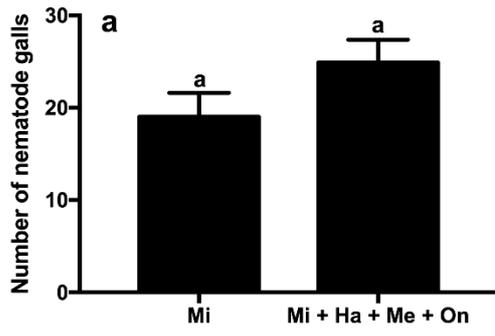
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423

424 **Figure 2**

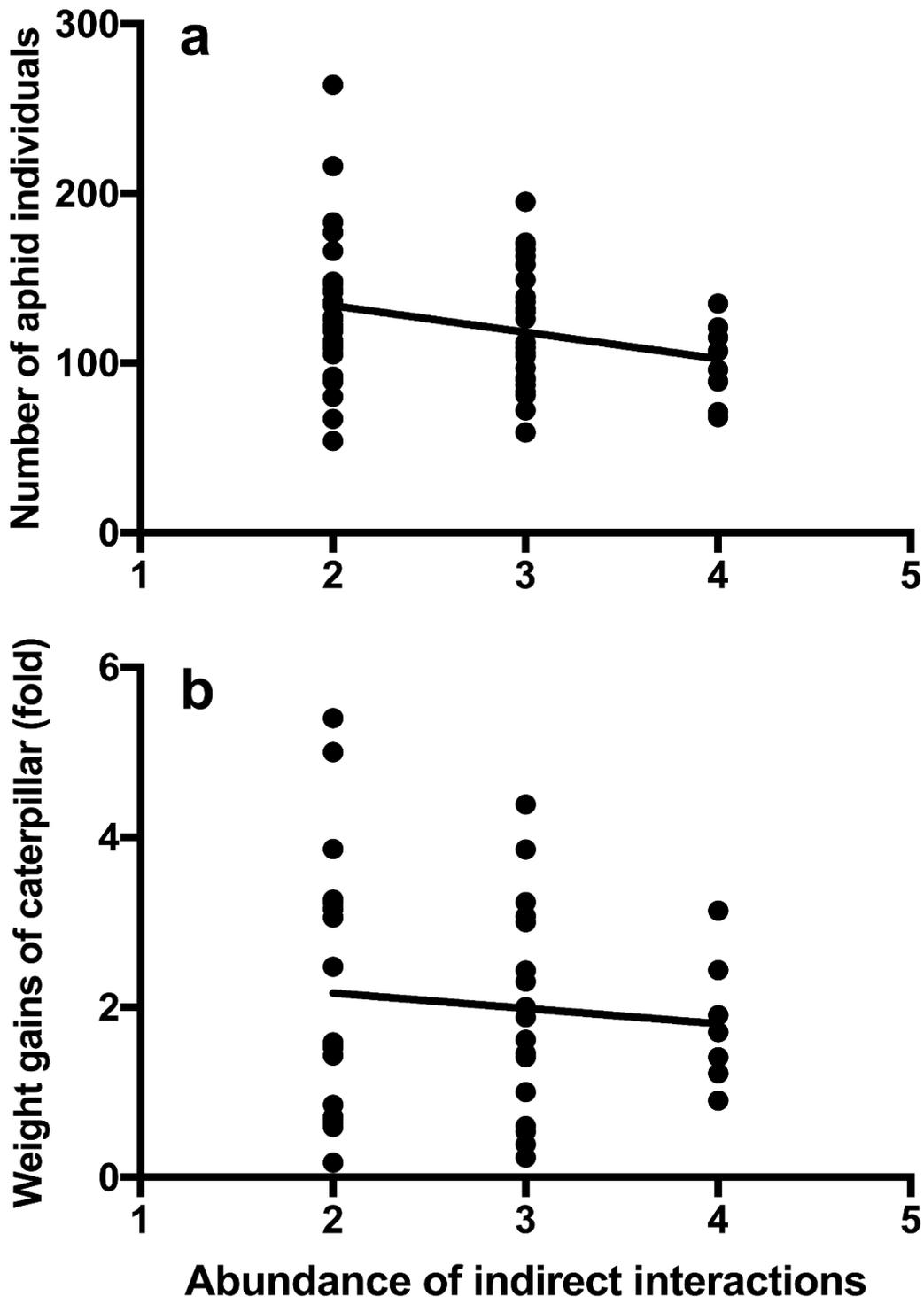
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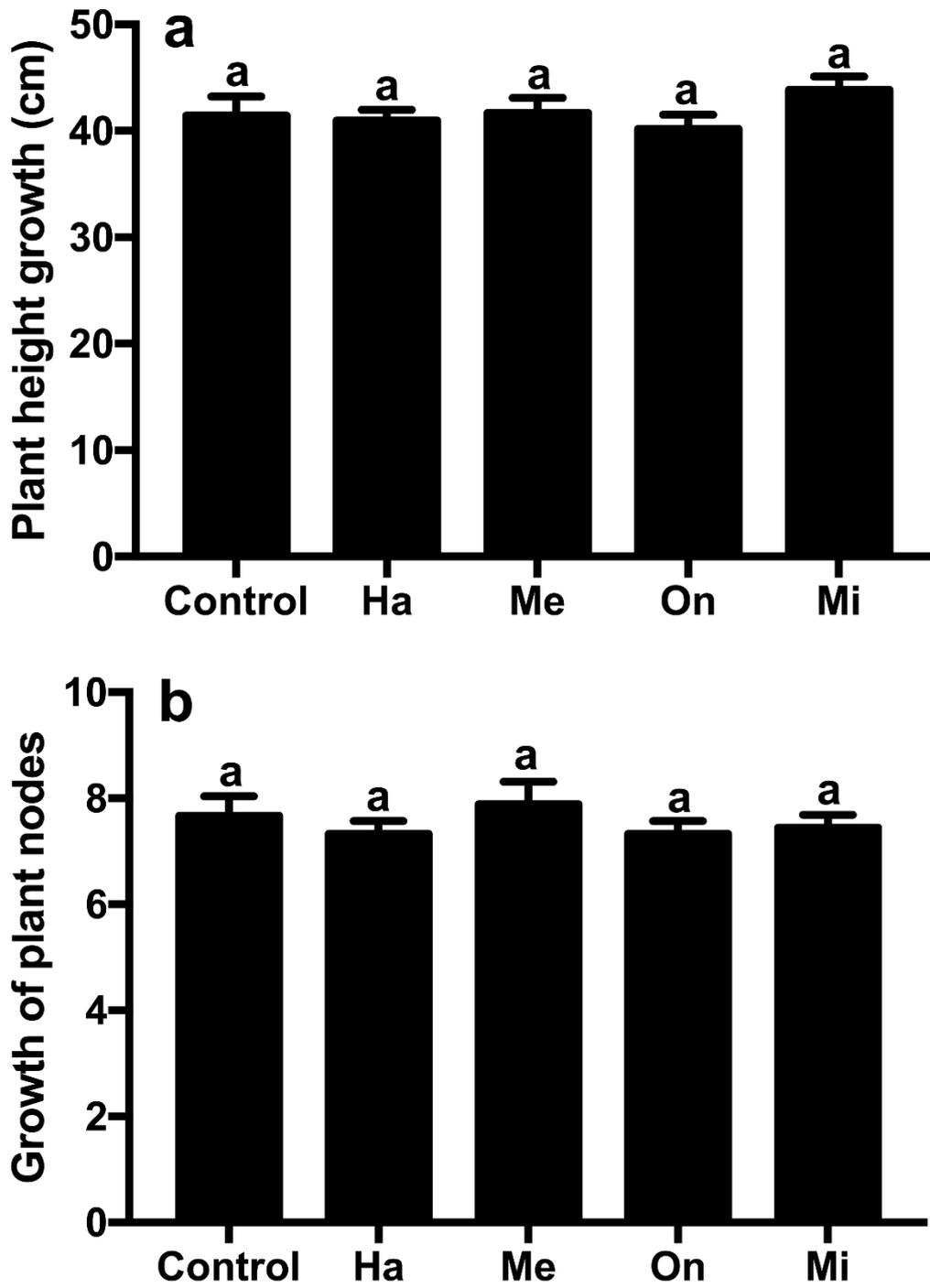
426

427 **Figure 3**

428



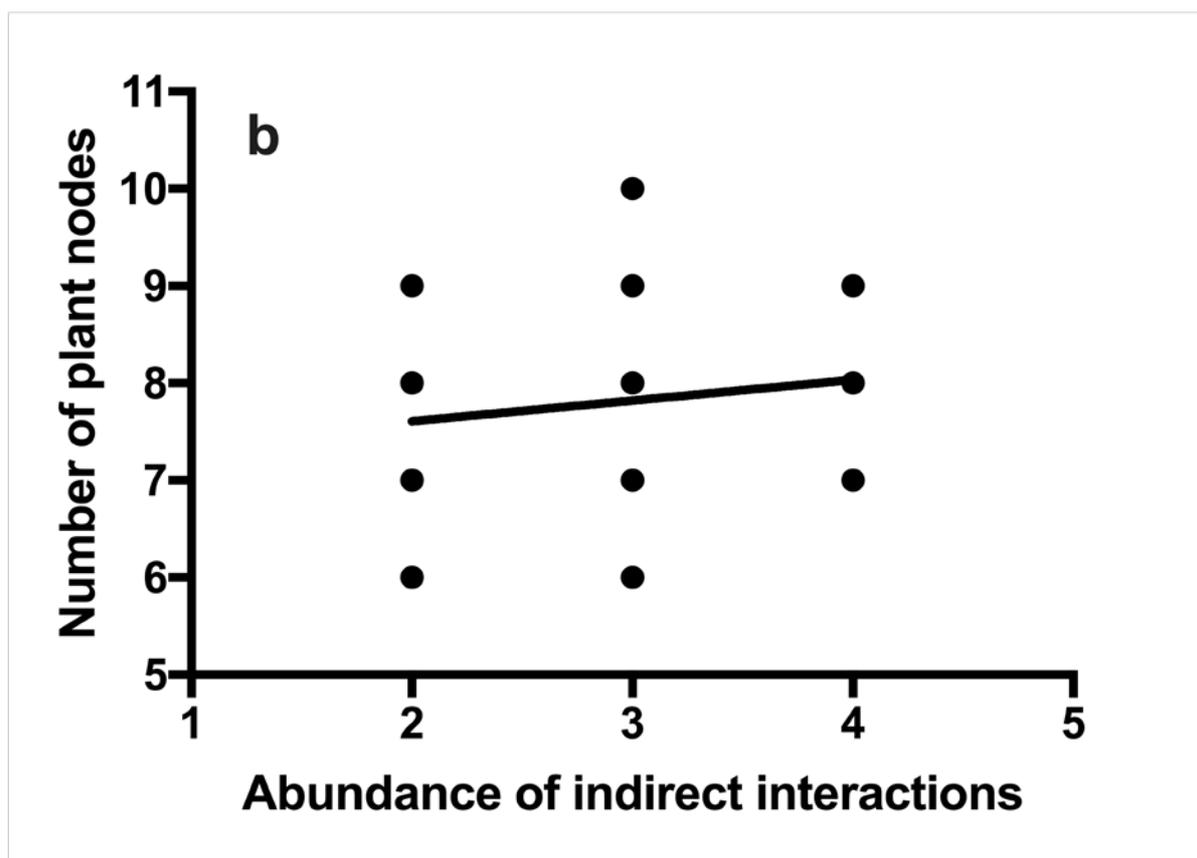
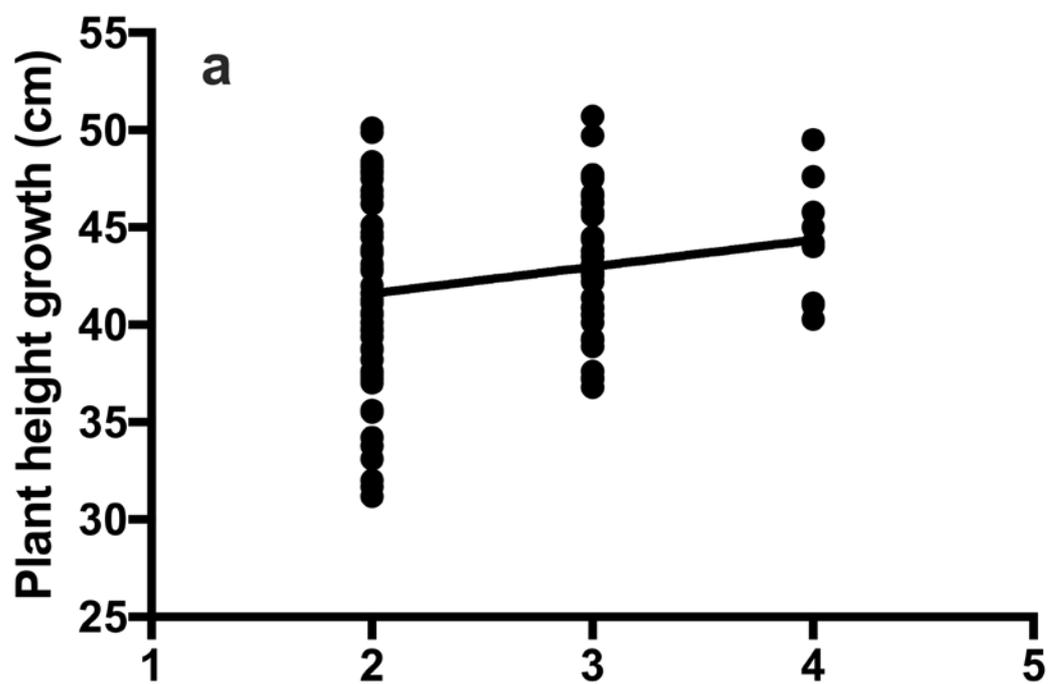
430 Figure 4



431

432 Figure 5

433



434

435 Figure 6

3. Sublethal effects of pesticides modulate interspecific interactions between the specialist aphid and the generalist aphid on soybeans

Article 3

Qu YY, Ullah F, Luo C, Monticelli LS, Lavoit AV, Gao XW, Song DL, Desneux N

Sublethal effects of beta-cypermethrin modulate interspecific interactions between the specialist aphid *Aphis glycines* and the generalist aphid *Aulacorthum solani* on soybeans

Ecotoxicology and Environmental Safety, accepted

1 **Sublethal effects of beta-cypermethrin modulate interspecific interactions between the**
2 **specialist aphid *Aphis glycines* and the generalist aphid *Aulacorthum solani* on soybeans**

3

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15

16

17

18 **Abstract**

19 In the agroecosystem, plant-pest interactions are the basic element to construct a complete food
20 web, which can be affected by both biotic and abiotic factors. In the current study, we aimed
21 at the sublethal effects of beta-cypermethrin on the interspecific interactions between the
22 specialist aphid *Aphis glycines* and the generalist aphid *Aulacorthum solani* on the soybean, by
23 measuring aphid life-history traits and host susceptibility. *A. glycines* had a higher fecundity
24 than *A. solani* on the soybean and they caused asymmetrically reproductive suppression on
25 each other. The sublethal concentration (LC₅ for *A. glycines*) of beta-cypermethrin used in this
26 study stimulated the reproduction of *A. glycines* but it did not have an influence on the fecundity
27 of *A. solani*. However, sublethal beta-cypermethrin caused a greater interspecific inhibition on
28 the fecundity of the two aphids species. Moreover, the two species of aphids had different
29 spatial distribution on soybean seedlings. *A. glycines* mainly resided on the stem of soybean
30 while *A. solani* preferred to colonizing soybean leaves. Sublethal pesticides drove *A. solani*
31 migrating from soybean leaves to stems no matter whether it suffered the interspecific
32 constraint or not. *A. glycines* significantly facilitated *A. solani* colonization on the soybean by
33 the induced host susceptibility, and vice versa. Nevertheless, such facilitated
34 colonization/induced susceptibility would be significantly impaired by the exposure to
35 sublethal beta-cypermethrin. Consequently, the interspecific interactions between specialist
36 and generalist aphids may be manipulated by the sublethal effects of beta-cypermethrin, via
37 altering aphid physiological and ecological traits and impairing the induced susceptibility of
38 soybeans.

39 **Key words:** sublethal effect, beta-cypermethrin, interspecific competition, *Aphis glycines*,
40 *Aulacorthum solani*, induced host susceptibility

41 **1.Introduction**

42 Aphids (Hemiptera: Aphididae) specialized in feeding plant sap, are one of the most destructive
43 pests on soybean *Glycine max* [L.] (Giordanengo et al., 2010; Hohenstein et al., 2019). High
44 population densities of aphids are achieved due to their parthenogenesis reproduction and the
45 short generation time. Virtually, aphids can colonize all the parts of soybean, consume
46 photoassimilates and alter the source-sink relationship (Hullé et al. 2020). In addition, they
47 could be also as vectors of various plant viruses causing significant quality decline and yield
48 loss in soybeans, due to inducing multiple viral diseases (Giordanengo et al., 2010; Nalam et
49 al., 2019; Powell et al., 2006; Ullah et al., 2019c). Soybean aphid, *Aphis glycines* Matsumura,
50 one of the specialist pests on soybean plants, has become a global pest in the last two decades
51 (Ragsdale et al., 2011). *Aphis glycines* can reduce soybean yield by up to 40% - 58% via
52 reduced quantity of soybean pods, smaller seed size, transmission of viruses such as *Soybean*
53 *mosaic virus*, and *Alfalfa mosaic virus*, and even potential facilitation to infestation of soybean
54 cyst nematodes in soybean roots (Hill et al., 2001; Qu et al., 2017; Ragsdale et al., 2007, 2011).
55 Foxglove aphid, *Aulacorthum (Acyrthosiphon) solani* Kaltenbach, native to Europe, has been
56 a cosmopolitan pest on a wide range of host plants (approximately 95 different plant species
57 from 25 families), such as pepper, potato, lettuce and soybean (Down et al., 1996; Jandricic et
58 al., 2010, 2014; Sanchez et al., 2007). *A. solani* infesting plants could cause leaf necrosis,
59 deformation of soybean grains, and even defoliation of the entire plants at high density due to
60 the toxicity of its salivary secretions to hosts (Sanchez et al., 2007). It is as well a viral vector
61 for various plant viruses and its honeydew benefits the fungal growth on leaves and thereby
62 obstructs photosynthesis of plants (Sato et al., 2013, 2014). All these problems may cause
63 significant yield loss up to 70-90% (Nagano et al., 2001; Sato et al., 2013).

64 To date, even though a variety of sustainable approaches have been exerted to control the
65 two species of aphids, including introduction of natural enemies (Costamagna et al., 2008;

66 Desneux and O'Neil, 2008; Desneux et al., 2006; 2009) and selection of aphid-resistance
67 soybean strains (Lee et al., 2015; Ragsdale et al., 2011), pesticide application is still at the
68 forefront in most countries due to the rapid reduction of aphid population density (Desneux et
69 al., 2007; Koch et al., 2018; Mohammed et al., 2018; Ullah et al., 2019a). Beta-cypermethrin,
70 one of the pyrethroids pesticides with a broad of insecticide spectrum, has been widely used to
71 control aphids in the soybean fields depending on its high efficacy and long persistence with a
72 stable formation (Hodgson et al., 2012; Ragsdale et al., 2011; Qu et al., 2017). In addition to
73 the lethal effects of beta-cypermethrin, pests may be exposed to sublethal concentrations with
74 the degradation of beta-cypermethrin in fields after initial application (Desneux et al., 2005;
75 Qu et al., 2017). Furthermore, the sublethal effects (Desneux et al., 2007; Jam and Saber, 2018;
76 Passos et al., 2018) of beta-cypermethrin involving physiological and behavioral alterations on
77 the individuals that survived post-exposure to sublethal concentrations have been documented
78 on some aphids (Gao et al., 2008; Wang et al., 2014; Zuo et al., 2016). Sublethal concentrations
79 (LC₅ and LC₁₅) of beta-cypermethrin decreased adult longevity and oviposition period of *A.*
80 *glycines* but they had reversed effects on adult fecundity, i.e. the reproduction of adults was
81 stimulated when *A. glycines* was exposed to LC₅ whereas it was suppressed at LC₁₅ in our
82 previous study (Qu et al., 2017).

83 Plants are commonly attacked by various pests with different feeding habits
84 simultaneously or successively (Bompard et al., 2013; Jaworski et al., 2015; Mohammed et al.,
85 2019; Desneux et al., 2019). Induced defenses mainly depending on phytohormonal signaling
86 pathways including salicylic acid (SA), jasmonic acid (JA), ethylene (ET) would be activated
87 locally and systemically after insects feeding on the plants and thereby result in the downstream
88 defenses with production of a variety of plant secondary metabolites (Biere and Goverse, 2016;
89 Han et al. 2020; Kersch-Becker and Thaler, 2019; Nalam et al., 2019; War et al., 2018). Such
90 physiological or chemical alterations induced by the early arriving pests in plants may impact

91 the individual performance, preference and even population dynamics of the subsequent pests
92 on the same host (Kroes et al., 2016; Mouttet et al., 2011; 2013; Rechner et al., 2017; Stout et
93 al., 2006; Wondafrash et al., 2013). In addition to the interactions between signaling pathways
94 which are specifically induced by different feeding guilds of pests (Caarls et al., 2015; Stout et
95 al., 2006; Wondafrash et al., 2013), resource reallocation, modulation of source-sink
96 relationships in plants, and herbivory host specificity could as well shape the outcome of such
97 plant-mediated interspecific interactions between diverse plant consumers on the same host
98 (Biere and Goverse, 2016; Caarls et al., 2015; Hohenstein et al., 2019; Lemoine et al., 2013;
99 Studham and Macintosh, 2013). Moreover, there has been a long-standing hypothesis that
100 specialist and generalist pests interact with plants in different ways, including plant defenses
101 induced by them (Dussourd and Denno, 1994; Poelman et al., 2008). Therefore, the distinct
102 induction of plant defenses triggered by specialist and generalist aphids would contributed to
103 their interactions on soybeans. However, in order to tolerate plant defenses, constitutive and
104 induced adaption have been respectively developed for oligophagous and polyphagous insects
105 to respond to the insecticidal compounds produced by the hosts (Gatehouse, 2002). Along with
106 the co-evolution during the compatible interactions between aphids and their hosts, plant
107 defenses may be overcome and even inhibited due to aphid adaption, resulting in facilitation
108 to their colonization on the host (Giordanengo et al., 2010; Hogenhout and Bos, 2011; Yates
109 and Michel, 2018).

110 Plants act as the focus to connect various biotic factors (herbivores, viruses, bacteria, fungi,
111 and nematodes) and abiotic factors (water, nitrogen, temperature and pesticide) to form a
112 complex food web in agroecosystem. Moreover, multiple abiotic factors, e.g. water, nitrogen,
113 temperature, have been reported to impact the insect performance, plant-mediated interactions
114 between pests, and even the performance of a higher-trophic natural enemy (Blazheyski et al.,
115 2018; Dong et al., 2018; Han et al., 2014; 2016; 2019; Kutyniok and Müller, 2013). However,

116 the influence of chemical pesticides that play a significant role in agricultural industry on the
117 interspecific interactions between insects with different host specificity is unknown. Therefore,
118 in this study, investigations of aphid fecundity, spatial distribution and their host susceptibility
119 were conducted to explore how the sublethal effects of beta-cypermethrin shape interspecific
120 interactions between the specialist aphid *A. glycinis* and the generalist aphid *A. solani* on the
121 soybean plants. This may contribute to comprehensively understanding soybean-aphid-
122 pesticide interactions and optimizing the aphid integrated management in soybean fields.

123

124 **2. Materials and Methods**

125

126 *2.1 Study insects*

127

128 The laboratory colonies of *A. glycinis* and *A. solani* were respectively established from
129 apterous individuals that were collected from the soybean fields in Langfang (Hebei province,
130 China) June 2007 and in Harbin (Heilongjiang province, China) June 2014. The two colonies
131 were maintained by continuous supply of insecticide-free soybean seedlings in climate
132 chambers, at $25 \pm 2^{\circ}\text{C}$, $60 \pm 10\%$ RH (relative humidity), L17: D7 photoperiod (Qu et al., 2015,
133 2017).

134

135 *2.2 Insecticide and acute toxicity assessment*

136

137 Analytical grade beta-cypermethrin (95%) was purchased from Jiangsu Pesticide
138 Research Institute Co., Ltd (China) and a 2000 mg/L stock solution was obtained with acetone
139 solvent. Both insect-dipping (NY/T 1154.6-2008) and leaf-dipping methods (NY/T 1154.14-
140 2008) were applied in bioassays to evaluate the toxicity of beta-cypermethrin on *A. glycinis*

141 and *A. solani* depending on the Agricultural Industry standard of the People's Republic of China.
142 Two concentration gradients of beta-cypermethrin (obtained from pretests as well as our
143 previous study) from the stock solution diluted by 0.005% (v/v) Triton X-100 (a non-ionic
144 surfactant) were used in the bioassays on *A. glycines* (1, 10, 25, 50 and 100 µg/L) and *A. solani*
145 (10, 100, 250, 500 and 1000 µg/L), respectively (Qu et al., 2017). Leaf discs (20 mm diameter)
146 from insecticide-free soybean leaves and the 3rd-instar apterous nymphs were dipped into the
147 corresponding concentrations of beta-cypermethrin solution for 10 s, and then placed in
148 shadow to air dry for 1 hour. One leaf disc and twenty aphids per replicate exposed to the same
149 concentration of beta-cypermethrin were transferred into one well of a 12-well tissue-culture
150 plate which contained 2% agar on the bottom and then covered with a filter paper strip
151 (Hangzhou, China). Control solution for *A. glycines* and *A. solani* contained 0.005% (v/v)
152 Triton X-100 (a non-ionic surfactant) and 0.005% or 0.05% acetone, respectively, which was
153 equal to the concentration of acetone in the highest treatment solution. There were four
154 replicates for each concentration of beta-cypermethrin and control and the mortality of aphids
155 was assessed after 24 hours exposed to each solution. Aphids were presumed dead when there
156 was no reflex movement, i.e. no leg moved when they were touched with a fine brush (Moore
157 et al., 1996). All the experimental setups were maintained in the climate chambers as the same
158 condition described above. The concentration-mortality regression equation (Abbott, 1925)
159 and LC₅, LC₅₀, LC₉₀ values of beta-cypermethrin on *A. glycines* and *A. solani*, were
160 respectively calculated for the further experiments.

161

162 2.3 Sublethal concentration assessment

163

164 The sublethal concentration of beta-cypermethrin to be tested was selected according to
165 the definition provided by Desneux et al. (2007) who defined sublethal concentrations as those

166 not inducing statistically significant mortality when compared to a control group. We selected
167 the LC₅ as sublethal concentration following the same paradigm than previous studies which
168 assessed sublethal effects of pesticides on arthropods (e.g. Qu et al. 2015; 2017). The
169 insecticide exposure was carried out using insect-dipping and leaf-dipping methods as
170 described above (section *Insecticide and acute toxicity assessment*) and the sublethal
171 concentration of beta-cypermethrin tested was selected as 7 µg/L (see section 3.1, below). Leaf
172 discs (20 mm diameter) and 3rd-instar nymphs of each species were separately dipped into 7
173 µg/L beta-cypermethrin for 10 s and then they were placed in shadow to air dry for 1 hour. One
174 leaf disc and twenty aphids treated by the sublethal concentration of beta-cypermethrin (7 µg/L)
175 or control solution (0.005% Triton X-100) were introduced into one well of the tissue-culture
176 plate. Two percent of the agar was pre-placed at the bottom of the well and then it was covered
177 with a filter paper strip (Hangzhou, China). Twenty-four-hour post-exposure to the low
178 concentration of beta-cypermethrin (7 µg/L), mortality of the two species of aphids was
179 respectively measured as well as that observed in control solution. The treatment and control
180 of the two species were both replicated four times.

181

182 *2.4 Effects of sublethal beta-cypermethrin and interspecific constraint on aphid fecundity and* 183 *spatial distribution on the soybean plants*

184

185 The influence of sublethal concentration of beta-cypermethrin and interspecific
186 interaction on aphids was designed with a two-factor experiment. Experiments followed a full
187 factorial design (Table 1). The impacts of interspecific constraint on aphids was assessed with
188 cross-infestation on the soybean plant, i.e. pre-infestation by a first attacker affects
189 performance of the second attacker. The sublethal effects design used control plants
190 with/without the first attackers, i.e. without any application of sublethal pesticide, and treated

191 plants with/without the first attackers, i.e. plants with/without the first attackers exposed to
192 pesticide.

193 When the soybean plant was only attacked by one aphid species (the second attacker), the
194 15-day-old soybean seedling was dipped into the sublethal concentration (7 µg/L) of beta-
195 cypermethrin for 10s, and then placed in shadow to air dry for 1 hour. Five days later, five
196 apterous adults of *A. solani* (in treatment A2)/ *A. glycines* (in treatment B2) were settled on the
197 top leaf (the first trifoliolate leaf) of the soybean seedling. After four days, the individual numbers
198 of the only attacker (*A. solani*/ *A. glycines*) on leaves and stems were measured, respectively.
199 Treatments A1 and B1 where the same-age soybean seedlings were exposed to 0.005% Triton
200 X-100 were considered as the control for each aphid species, i.e. not the pesticide exposure nor
201 the pre-infestation in the treatments A1 and B1.

202 When the soybean was infested by two different attackers, the two species of aphids were
203 introduced on the soybean in sequence. For instance, when *A. glycines* was the first attacker
204 (in treatment A4) on soybean plants, both 15-day-old soybean seedlings and 3rd-instar *A.*
205 *glycines* apterous nymphs were treated by the sublethal concentration of beta-cypermethrin for
206 10s, and then placed in shadow to air dry for 1 hour. Five survived *A. glycines* nymphs were
207 introduced on the unifoliolate leaf for five days, and then five *A. solani* apterous adults were
208 settled on the top leaf (the first trifoliolate leaf) of the soybean plants. After four days, the
209 individual numbers of *A. solani* on leaves and stems were measured, respectively. Experiments
210 followed the same scenario as described above when *A. solani* was the first attacker (in
211 treatment B4) arriving on soybean plants. The same-age soybean seedlings and the first attacker
212 (*A. glycines*/ *A. solani*) were treated by the Triton X-100 for 10 s in treatments A3 and B3,
213 respectively. Thereafter, the same procedure as that in treatments A4 and B4 was conducted
214 for both infestation and measurement of the second attacker in treatments A3 and B3.

215 One soybean seedling represents one replicate, which was separately caged and
216 maintained in climate chambers, at $25 \pm 2^{\circ}\text{C}$, $60 \pm 10\%$ RH (relative humidity), L17: D7
217 photoperiod. Each treatment was replicated 20 times.

218

219 *2.5 Effects of sublethal beta-cypermethrin and interspecific constraint on susceptibility of* 220 *soybean plants to aphids*

221

222 Four kinds of 15-day-old soybean plants, i.e. (1) clean plants without pesticide application
223 and pre-infestation by aphids, (2) plants exposed to sublethal concentration of beta-
224 cypermethrin for 10s, (3) plants infested by the first attackers (five 3rd-instar apterous nymph
225 aphids), (4) plants with infestation of the first attackers (five 3rd-instar apterous nymph)
226 together exposed to sublethal concentration of beta-cypermethrin for 10s, were prepared to
227 place in the climate chambers for 5 days. Thereafter, one leaflet from each treatment was
228 harvested for preparation of leaf discs to assess the plant susceptibility to aphids. Sixty the
229 second attackers (apterous adult aphids after a starvation period of 3 hours) were introduced to
230 the center of Petri dish surround with four leaf discs (one of each type). The number of aphids
231 on each leaf disc was recorded after they moved, fed and settled on each leaf for 1 hour with
232 15 replications.

233

234 *2.6 Data analysis*

235

236 The concentration-mortality regression equation and values of LC_{90} , LC_{50} and LC_5 were
237 calculated with a Probit analysis. All the data were normal by Kolmogorov-Smirnov test.
238 Mortality of each species of nymph aphids exposed to low concentration of beta-cypermethrin
239 and control solution was analyzed by T-test to assess the sublethal concentration of beta-

240 cypermethrin. The impacts of sublethal beta-cypermethrin and interspecific constraint on
241 fecundity, spatial distribution on soybeans and plant susceptibility to the second attackers were
242 analyzed with the two-way ANOVA (analysis of variance) in a generalized linear model
243 (GLM). Thereafter, the univariate analysis was carried out and a Tukey's HSD test for multiple
244 comparisons was processed to compare the difference on aphid fecundity, spatial distribution
245 and plant susceptibility among different treatments. All the analyses were performed with the
246 SPSS 25.0 software.

247

248 **3.Results**

249

250 *3.1 Acute toxicity of beta-cypermethrin on A. glycines and A. solani*

251

252 The results of the log-probit regression analyses between the beta-cypermethrin
253 concentrations tested and the mortality recorded in *A. glycines* and *A. solani* individuals are
254 reported in Table 2. The beta-cypermethrin was more toxic to *A. glycines* than on *A. solani* (the
255 LC₅₀ value was nearly 10 times lower for *A. glycines*). Therefore, the lowest LC₅ value, i.e.
256 the one causing 5% in mortality in *A. glycines* (7 µg/L), was selected as the tested sublethal
257 concentration for the next assessments which implied exposing both species to a single
258 sublethal concentration on a shared plant. This enabled to expose both aphid species
259 simultaneously to a concentration not inducing significant mortality in exposed individuals
260 when compared to a control (i.e. sublethal concentration according to Desneux et al. 2007).

261

262 *3.2 Assessment of sublethal concentration on both A. glycines and A. solani*

263

264 The exposure to 7 µg/L beta-cypermethrin induced $7 \pm 1.22\%$ and $2 \pm 1.22\%$ mortality in
265 *A. glycines* and *A. solani*, respectively. As expected, these mortality values were not
266 significantly different than the mortality values recorded in respective controls (*A. glycines*: 5
267 $\pm 1.58\%$, $P = 0.347$; *A. solani*: $1 \pm 1\%$, $P = 0.545$), and it confirmed that 7 µg/L could be
268 considered as one of the sublethal concentrations of beta-cypermethrin on these two aphids.

269

270 3.3 Effects on aphid fecundity

271

272 As shown in Table 3, interspecific constraint had significant influences on the fecundity
273 of both *A. solani* ($F_{1,76} = 8.724$; $P = 0.004$) and *A. glycines* ($F_{1,72} = 76.393$; $P < 0.001$), whose
274 fecundity was as well affected by the interaction of interspecific constraint and pesticide ($F_{1,72}$
275 $= 9.861$; $P = 0.002$). Interspecific constraint didn't change *A. solani* fecundity without exposure
276 to sublethal concentration of beta-cypermethrin while it decreased the reproduction of *A. solani*
277 on the soybean plant which was treated by sublethal pesticide ($F_{3,76} = 3.389$; $P = 0.022$; Fig.1).
278 However, the fecundity of *A. glycines* was significantly reduced by the interspecific constraint
279 on both pesticide-treated and pesticide-free soybean plants. Moreover, sublethal pesticide
280 significantly increased the fertility of *A. glycines* on the soybean plants that were not pre-
281 infested by *A. solani*, but it didn't change the reproductive of *A. glycines* on the soybean plants
282 that were pre-infested by *A. solani* ($F_{3,72} = 28.247$; $P < 0.001$; Fig.1).

283

284 3.4 Effects on aphid spatial distribution on soybean plants

285

286 The percentage of *A. solani* individuals settling on the soybean leaf was significantly
287 impacted by pesticide ($F_{1,76} = 13.678$; $P < 0.001$; Table 3) while the distribution of *A. glycines*
288 on the soybean plant was distinctly affected by both interspecific constraint ($F_{1,70} = 19.703$; P

289 < 0.001; Table 3), pesticide ($F_{1,70} = 13.039$; $P = 0.001$; Table 3) and their interactions, i.e.
290 ‘interspecific constraint \times pesticide’ ($F_{1,70} = 24.705$; $P < 0.001$; Table 3). Exposure to sublethal
291 concentration of beta-cypermethrin significantly decreased the proportion of *A. solani*
292 colonizing on the soybean leaves no matter whether *A. solani* was under the stress of
293 interspecific interactions or not ($F_{3,76} = 4.615$; $P = 0.005$; Fig.2). However, the percentage of
294 *A. glycines* settling on pesticide-free soybean leaves was largely elevated by interspecific
295 constraint. Although pesticide treatment did not change the proportion of *A. glycines* on leaves
296 which were not pre-infested by *A. solani*, it significantly reduced the ratio of soybean aphids
297 colonizing on leaves with the foxglove aphid pre-infestation ($F_{3,70} = 18.957$; $P < 0.001$; Fig.2).

298

299 3.5 Effects on the plant susceptibility to aphids

300

301 Both the two factors, i.e. interspecific constraint ($F_{1,56} = 72.475$; $P < 0.001$ for *A. solani*;
302 $F_{1,56} = 63.603$; $P < 0.001$ for *A. glycines*) and pesticide ($F_{1,56} = 4.971$; $P = 0.03$ for *A. solani*;
303 $F_{1,56} = 7.826$; $P = 0.007$ for *A. glycines*), and their interactions ‘interspecific constraint \times
304 pesticide’ ($F_{1,56} = 18.992$; $P < 0.001$ for *A. solani*; $F_{1,56} = 18.496$; $P < 0.001$ for *A. glycines*)
305 had significant influences on the susceptibility of soybean plants to *A. glycines* and *A. solani*
306 (Table 3). Plant susceptibility to *A. solani* and *A. glycines* was both increased by the
307 interspecific constraint no matter whether they were exposed to sublethal concentration of beta-
308 cypermethrin or not. Pesticides did not change the plant’s susceptibility to *A. solani* and *A.*
309 *glycines* when aphids didn’t suffer the interspecific constraint, but the host susceptibility to the
310 two species of aphids that were subjected to interspecific constraint was significantly reduced
311 by pesticide treatment ($F_{3,56} = 32.146$; $P < 0.001$ for *A. solani*; $F_{3,56} = 29.975$; $P < 0.001$ for *A.*
312 *glycines*; Fig.2).

313

314 4.Discussion

315

316 In our study, we investigated the effects of both sublethal beta-cypermethrin (abiotic stress)
317 and interspecific constraint (biotic stress) on *A. glycines* and *A. solani* fecundity, spatial
318 distribution on soybeans and the plant susceptibility to these two species of aphids. The mutual
319 suppression on fecundity was detected in the interspecific interactions between *A. glycines* and
320 *A. solani*, which may be due to the induced plant defenses against the two species of aphids
321 (Hohenstein et al., 2019; Sato et al., 2014; Studham and Macintosh, 2013). Induced plant
322 responses triggered by *A. glycines* were mainly attributed to the JA signaling pathway which
323 may cause the accumulation of isoflavonoids that act as toxins against the subsequent aphids
324 on the soybean (Hohenstein et al., 2019). Moreover, after inoculation of *A. solani* on soybean
325 leaves, there were yellow blotches surrounding the feeding location, which was accompanied
326 by the decreased concentrations of citrate and amino acids, leading to the reduction of plant
327 quality for the subsequent aphids (Sato et al., 2013, 2014). The stress of sublethal beta-
328 cypermethrin increased the interspecific inhibition rate on *A. solani* fecundity from 13.4% to
329 26% that was calculated by the offspring numbers produced by each *A. solani* adult with an
330 equation of $(A2 - A4) / A2$ (Table 1; Fig.1). Moreover, the suppression rate on *A. glycines*
331 reproduction caused by the interspecific constraint was as well double enhanced from 34.36%
332 to 60.32% with exposure to sublethal concentration of the pesticide. Consequently, it indicates
333 sublethal pesticides can multiply the inhibition on aphid abundance caused by interspecific
334 competition. The mechanism(s) underneath pesticide-accelerated interspecific competitions
335 between the two species of aphids could be as a result of more energy spent to detoxify
336 pesticide and to migrate within plants, therefore causing the further failure of the second
337 attackers in interspecific interactions (Jager et al., 2013; Qu et al., 2017). In addition, the
338 suppression rate on *A. glycines* fecundity caused by the interspecific constraint was twice more

339 than that on *A. solani* fertility, which may be likely linked to the bigger biomass and body size
340 of *A. solani* as well as its higher spatial niches on soybean plants. *A. solani* mainly resides on
341 soybean leaves that are the more valuable source tissues to synthesize photosynthates.
342 Moreover, infestation by aphids may alter the source-sink dynamics of phloem transport via
343 the resource sequestration to secure the local resources for *A. solani* on leaves, thereby reducing
344 the carbohydrate supply to *A. glycines* that mainly settles on stems (Biere and Goverse, 2016;
345 Lemoine et al., 2013). In addition, the failure of *A. glycines* in the interspecific interactions
346 with *A. solani* may be as well attributable to the adaption malfunction of the specialist to some
347 new insecticidal compounds induced by the generalist aphid that can successfully colonize a
348 wide range of host plants (Gatehouse, 2002). However, compared to the generalist aphid *A.*
349 *solani* on the soybean plants, *A. glycines* as the specialist consumer has evolved more
350 physiological adaptations to cope with the plant constitutive defenses, e.g. the detoxification or
351 the sequestration of poisons (Ali and Agrawal, 2012; Barrett and Heil, 2012; Florent, 2013).
352 This may be responsible for the higher fertility performance of *A. glycines* on infestation-free
353 soybean plants. The sublethal concentration (LC₅ of beta-cypermethrin on *A. glycines*) used in
354 this study significantly facilitated the reproduction of *A. glycines* on clean soybean plants
355 (soybeans were only infested by one attacker, i.e. the second attacker, in treatments B1 and
356 B2), which was consistent with the hormesis on soybean aphid fecundity in our previous study
357 (Qu et al., 2017). However, the reversed/suppressed impact caused by sublethal beta-
358 cypermethrin on soybean aphid fecundity on the hosts pre-infested by *A. solani* may be likely
359 associated to the niche encroachment of *A. solani* that transferred from leaves to stem post-
360 exposure to the pesticide. Nevertheless, no influence was detected on the fecundity of *A. solani*
361 post-exposure to beta-cypermethrin. It may be due to the higher resistance of *A. solani* to this
362 pesticide and the concentration used in the test (LC₁ on *A. solani*) may be too low to alter the
363 its reproduction (Qu et al., 2017; Ullah et al., 2019b; Wang et al., 2014).

364 Niche complementarity is a strategy to maintain compatible interactions when diverse
365 plant predator species consume the same plant host (Gable et al., 2012; Northfield et al., 2010).
366 As demonstrated in our result (Fig. 2), *A. solani* and *A. glycines* population had different spatial
367 distribution on the soybean plant, where the generalist predator, *A. solani* mainly colonize the
368 leaves and the specialist, *A. glycines* preferred settling on stems. The distinction of host
369 specificity and the different composition of phloem sap within the plants probably as well drive
370 their niche choices (Jakobs and Müller, 2019; Jakobs et al., 2019). In addition, aphids
371 considered as the important model insects to research species differentiation and speciation,
372 may have a predisposition to sympatric speciation (Berlocher and Feder, 2002; Dixon, 1998;
373 Powell et al., 2006). In the current study, soybean aphids were observed to largely moved from
374 the stem to soybean leaves when they were under the stress of interspecific competition (Fig.
375 2). Because pesticides are more accessible to *A. solani* on soybean leaves for the larger surface,
376 alarm pheromones could be released by *A. solani* to warn the conspecific individuals to evade
377 and move to stems. Moreover, irritated by pesticides (including pyrethroids), chemoreceptors
378 located on the surface of insect body may also cause a reflex action, leading to the movement
379 of herbivores away from the exposed places (Alzogaray and Zerba, 2001; Desneux et al., 2007).
380 In addition to the pesticide, the failure of *A. glycines* in interspecific interactions with *A. solani*
381 is the other factor to impact the niche construction of soybean aphids. Alterations of aphid
382 spatial distribution on the soybean plants may cause natural enemy to spend more time foraging
383 for preys, thereby leading to the efficiency decline of aphid biological control (Desneux et al.,
384 2007; Guedes et al., 2016; Ragsdale et al., 2011).

385 It should be declared that pest-induced plant responses are not always detrimental to pests
386 and in fact may induce host susceptibility to pests by manipulating the plant responses
387 (Gatehouse, 2002; Inbar et al., 1999; Studham and Macintosh, 2013). It has been documented
388 that soybean aphids can increase the suitability of their host to conspecifics depending on

389 feeding facilitation and obviation of resistance (Varenhorst et al., 2015; Yates and Michel,
390 2018). The induced susceptibility of hosts to interspecific aphids in our study may be as well
391 hypothesized that aphids block effective defenses in the compatible interaction via secreting
392 salivary effectors that can overcome the host defenses to facilitate susceptibility (Hohenstein
393 et al., 2019; Yates and Michel, 2018). Consequently, the leaves on soybean plants pre-infested
394 by one species of aphids were easier to be colonized by the other species of aphids but they
395 would be abandoned by aphids under the treatment of sublethal beta-cypermethrin. Aphids
396 circumvented the leaves exposed to beta-cypermethrin, which may be associated with the
397 repellent and antifeedant activities of pyrethroid, e.g. on *Apis mellifera* and *Aedes aegypti*
398 (Bandason, 2018; Bowman et al., 2018; Rieth and Levin, 1988). The survival of the infestation-
399 free leaves in face of aphid colonization implies that constitutive defense strategies are as well
400 employed by soybean plants in addition to the induced defenses. The constitutive defenses
401 could act as a barrier by lignification and production of feeding or egg deposition deterrents to
402 protect hosts from colonizing by insects (Bixenmann et al., 2016; Gatehouse, 2002; Mithöfer
403 and Maffei, 2017).

404

405 **5.Conclusion**

406

407 To conclude, compared to *A. solani*, the preference and performance of *A. glycines* were
408 easier to be affected by both the abiotic and biotic stress. Interspecific constraint inhibited the
409 fecundity of both *A. solani* and *A. glycines*, but sublethal concentration of beta-cypermethrin
410 had stimulatory effects only on the fecundity of *A. glycines* on the soybeans without pre-
411 infestation. Exposure to sublethal beta-cypermethrin distinctly drove *A. solani* migrating from
412 leaves to stems and also caused *A. glycines* to flocked to the stems when soybean plants were
413 pre-infested by *A. solani*. Interspecific constraint did not change the distribution of *A. solani*

414 colonizing on soybean plants yet lead to a transfer of *A. glycines* to pesticide-free soybean
415 leaves.

416 Furthermore, we constructed a network (Fig. 4) of soybean plants, aphids, and pesticides
417 to elucidate the negative but asymmetric interactions between the specialist aphid *A. glycines*
418 and the generalist aphid *A. solani* on the population, via their sympatric feeding on soybean
419 plants. The aphid reproduction was mutually inhibited while their colonization on the host was
420 facilitated by each other. Beta-cypermethrin, which is a commonly used pesticide for aphid
421 control in the soybean field but which has been recently reported to cause pest resurgence
422 associated with its sublethal effects, was firstly considered to assess its sublethal effects on the
423 aphid interspecific interactions between specialist and generalist aphids. Sublethal
424 concentration of beta-cypermethrin caused the acceleration of interspecific competition and the
425 reduction of aphid-induced susceptibility of hosts. All these results may contribute to fully
426 understanding the plant-aphid-pesticide interactions and expanding our knowledge of the side-
427 effects of pesticides used in the agro-system and thereby developing the integrated strategies
428 to control aphids in soybean fields.

429

430 **Acknowledgements**

431 This work was funded by the European Union's Horizon 2020 Research and Innovation
432 Program (Grant number: 6339999), the National Key Research and Development Program of
433 China (Grant number: 2016YFD0200500), the National Natural Science Foundation of China
434 (Grant number: 31272077), and China Scholarship Council.

435

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449

450 **Competing for financial interests:** the authors declare that no competing financial interests.

451

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683 **Table legends**

684

685 **Table 1. Full factorial experimental design on factors of sublethal beta-cypermethrin**
686 **and interspecific constraints**

| Treatment | plant | The 1 st attacker | Beta-cypermethrin | The 2 nd attacker |
|-----------|------------------|------------------------------|-------------------|------------------------------|
| A1 | Soybean seedling | No | No | <i>A. solani</i> |
| A2 | Soybean seedling | No | Yes | <i>A. solani</i> |
| A3 | Soybean seedling | <i>A. glycines</i> | No | <i>A. solani</i> |
| A4 | Soybean seedling | <i>A. glycines</i> | Yes | <i>A. solani</i> |
| B1 | Soybean seedling | No | No | <i>A. glycines</i> |
| B2 | Soybean seedling | No | Yes | <i>A. glycines</i> |
| B3 | Soybean seedling | <i>A. solani</i> | No | <i>A. glycines</i> |
| B4 | Soybean seedling | <i>A. solani</i> | Yes | <i>A. glycines</i> |

687 Both soybean seedlings and the 1st attackers were treated by the sublethal concentration of
688 beta-cypermethrin in this study.

689

690 **Table 2. Toxicity of beta-cypermethrin on the 3rd nymphs of *A. solani* and *A. glycines***

| Aphid | concentration-response | χ^2 | df | P | LC ₅ / μ g/L | LC ₅₀ / μ g/L | LC ₉₀ / μ g/L |
|--------------------|------------------------|----------|----|-------|-----------------------------|------------------------------|------------------------------|
| | regression equation | | | | (95% CI) | (95% CI) | (95% CI) |
| <i>A. glycines</i> | Y = 3.212 X+5.268 | 7.387 | 18 | 0.986 | 7 (5 - 9) | 23 (20 - 26) | 57 (48 - 73) |
| <i>A. solani</i> | Y = 3.013 X+2.099 | 23.561 | 18 | 0.170 | 57 (38 - 76) | 201 (171 -232) | 536 (446 - 686) |

691 95% CI indicates 95% confidence interval; the variable X is transformed using a log base of

692 10

693

694

695 **Table 3. Parameters of two-factor analysis of effects on aphid fecundity, spatial**
 696 **distribution on soybeans and the plant susceptibility to *A. glycines* and *A. solani***

| Effects | Factors | Effects on <i>A. solani</i> | | Effects on <i>A. glycines</i> | |
|---|---|-----------------------------|----------------|-------------------------------|----------------|
| | | F | <i>P</i> value | F | <i>P</i> value |
| Fecundity | Interspecific constraint | 8.724 | 0.004* | 76.393 | <0.001* |
| | Pesticide | 0.252 | 0.617 | 0.068 | 0.795 |
| | Interspecific constraint × pesticide | 1.190 | 0.279 | 9.861 | 0.002* |
| Spatial distribution on soybeans | Interspecific constraint | 0.153 | 0.697 | 19.703 | <0.001* |
| | Pesticide | 13.678 | <0.001* | 13.039 | 0.001* |
| | Interspecific constraint × pesticide | 0.014 | 0.907 | 24.705 | <0.001* |
| Plant susceptibility | Interspecific constraint | 72.475 | <0.001* | 63.603 | <0.001* |
| | Pesticide | 4.971 | 0.03* | 7.826 | 0.007* |
| | Interspecific constraint × pesticide | 18.992 | <0.001* | 18.496 | <0.001* |

697 *P* value followed by asterisk (*) signifies that the factor has the significantly effect on aphid
 698 fecundity, spatial distribution or host susceptibility to the second arriving aphids

699 **Figure legends**

700 **Fig. 1 The offspring (Mean number \pm SEM) produced by each *A. solani* (group A) and *A.***
701 ***glycines* (group B) adult on different treatment soybean plants.**

702 Different letters in each group indicate significant difference at $P < 0.05$.

703

704 **Fig. 2 Distribution (Mean percentage \pm SEM) of *A. solani* (group A) and *A. glycines***
705 **(group B) on the leaves of soybean plants in different treatments.**

706 Different letters in each group indicate significant difference at $P < 0.05$.

707

708 **Fig. 3 Colonization (Mean percentage \pm SEM) of *A. solani* (group A) and *A. glycines***
709 **(group B) on different treatment leaves.**

710 Different letters in each group indicate significant difference at $P < 0.05$.

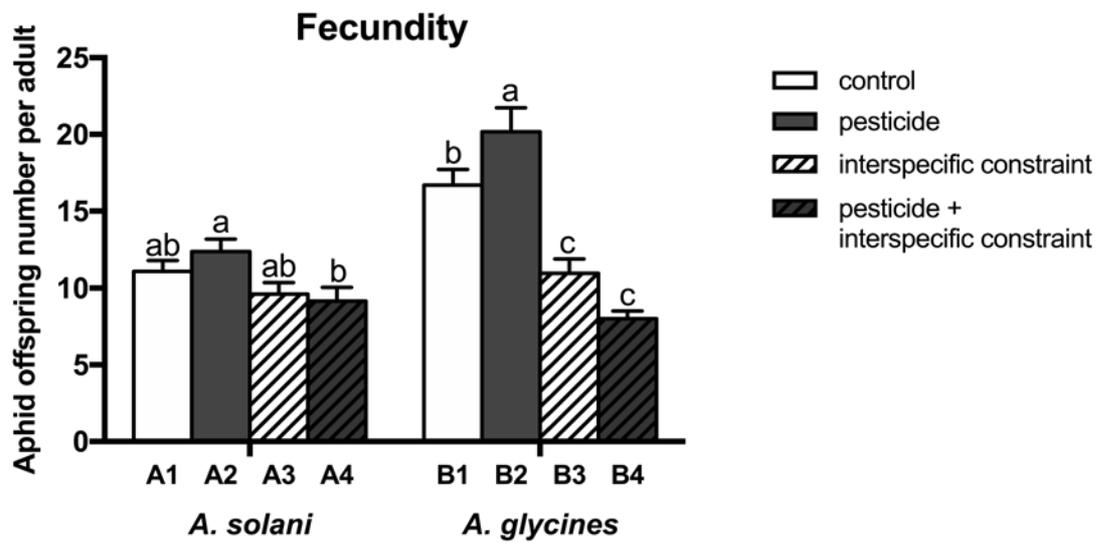
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712 **Fig. 4 A network of soybean plant-specialist aphid-generalist aphid-pesticide interactions.**

713 *A. solani*, the generalist aphid primarily settling on soybean leaves, had a higher spatial niche
714 than *A. glycines*, the specialist aphid that mainly colonizes on soybean stem. Aphids as the
715 consumer of phloem sap were supported by the soybean plants for food resource, but on the
716 other hand, aphids were against by plant defensive metabolites. When *A. glycines* and *A. solani*
717 attack soybean plants in sequence, the fecundity of the subsequent arrivers was inhibited by
718 the interspecific constraint. Moreover, interspecific constrain caused stronger suppression on
719 the reproductive of soybean aphids, compared to foxglove aphids. However, pre-infestation by
720 one species of aphids facilitated subsequent aphids' colonization on the host by induced plant
721 susceptibility to aphids. Beta-cypermethrin as a commonly used pesticide to control aphid
722 population stimulated the reproduction of *A. glycines* at a sublethal concentration of 7 $\mu\text{g/L}$,
723 but it did not alter the fecundity of *A. solani* at this concentration. In addition, exposure to the

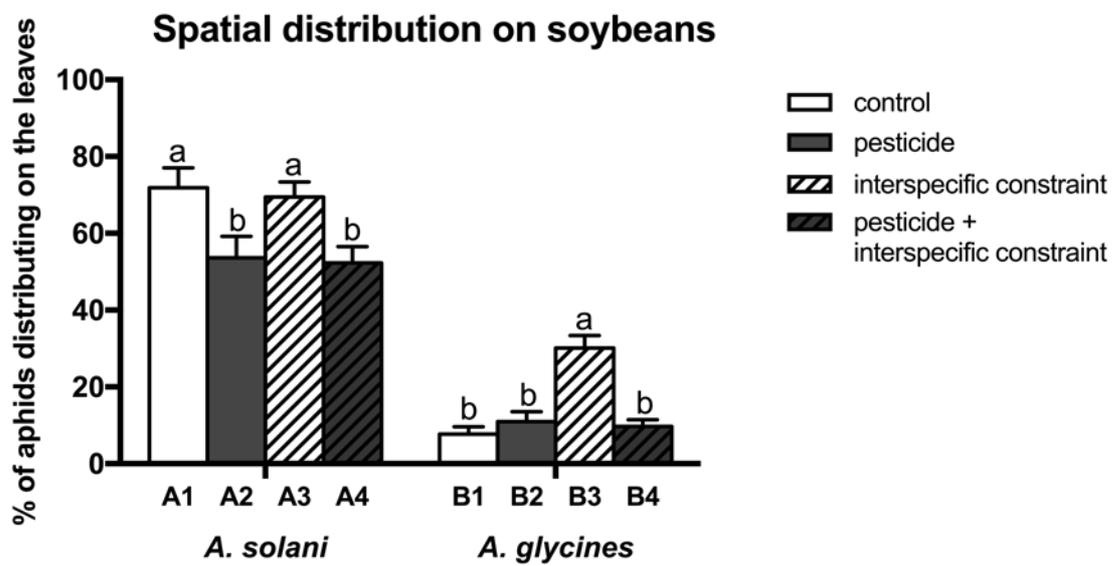
724 sublethal beta-cypermethrin caused a stronger interspecific suppression on the fecundity of the
725 two species of aphids, but it impaired the colonization facilitation caused by hetero-specific
726 aphids to subsequent arriving aphid species. Positive effects are illustrated by green arrows,
727 while negative effects are illustrated by red lines with block at the end. Two block indicate a
728 stronger inhibiting impact on fecundity of *A. glycines* in the interspecific competition,
729 compared to *A. solani*. Solid lines with block/arrow at the end indicate direct effects on
730 plants/aphids but dashed lines indicate interspecific interactions and indirect modulation on
731 interactions.

732 Fig. 1



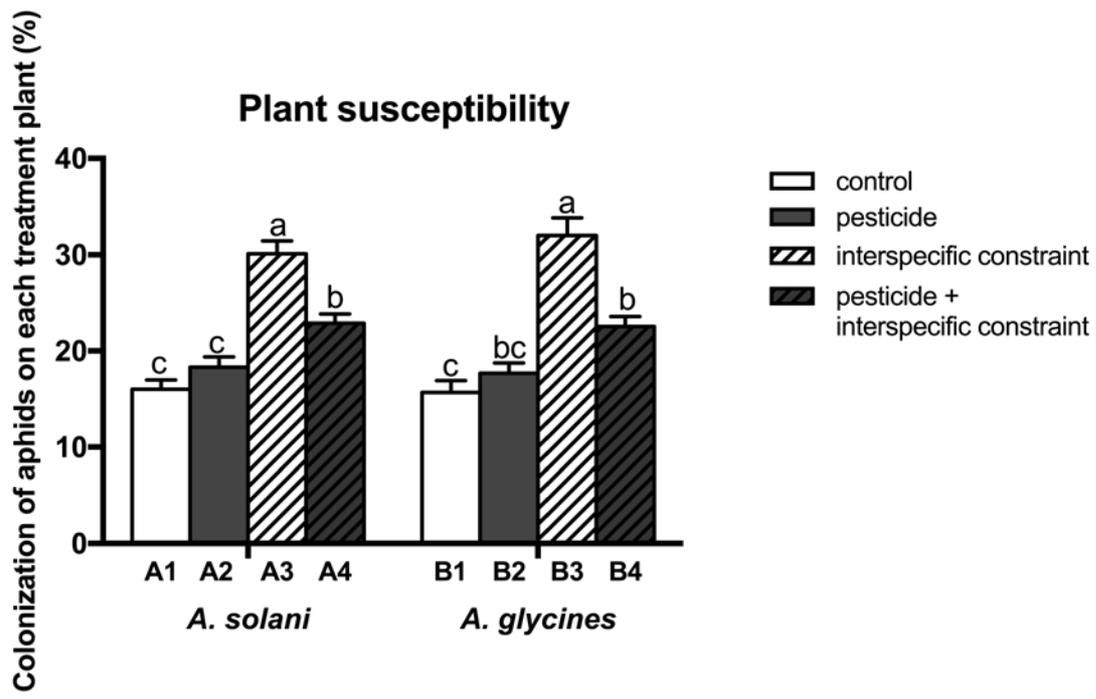
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734 Fig. 2



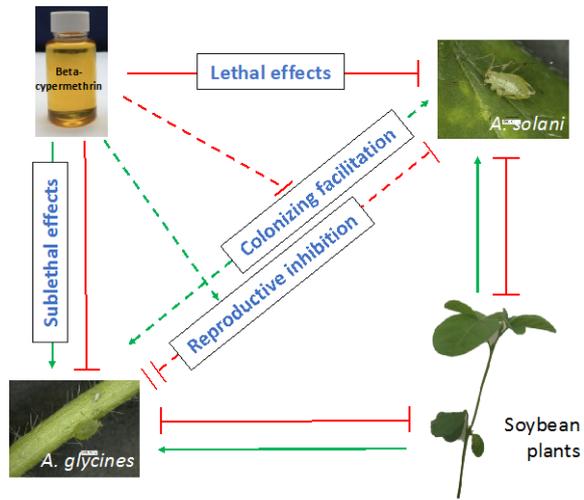
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736 **Fig. 3**



737

738 **Fig. 4**



739

CHAPTER 4. INFLUENCES OF ABOVE-BELOW GROUND INDIRECT INTERACTIONS ON *MACROSIPHUM EUPHORBIAE* POPULATION DYNAMIC AND THE BIOLOGICAL CONTROL

1. Introduction

In nature, plants are one of basic parts of complex food webs that include direct as well as indirect interactions among various organisms which are either enemies or allies (Kant et al., 2015). When plants are confronted with massive biotic threats, they can mediate indirect interactions among multiple pests via plant induced defenses not only within the same organ but also cross-organ of the plant (Biere and Goverse, 2016). The preference and performance of herbivore individuals on the host would be impacted by such indirect interactions (Biere and Goverse, 2016). Furthermore, population dynamics of insects would be also affected via the alteration in individual behaviors and/or performances, when they subsequently respond to the plant defenses triggered by the early season attackers (McCarville et al., 2012; Wondafrash et al., 2013). The host would be manipulated by all their consumers together, while attacked by multiple pests on different organs of the plants.

During recent decades, studies on the interactions among plants and other organisms has developed from the investigation of relatively simple interactions between one plant and one pest, to that of intricate interactions among one plant and multiple pests, and to that of more complex multi-trophic interactions (Janssen et al., 1998; Poelman et al., 2012; Price et al., 1980). Plant defenses, the main factor mediating such interactions above, include traits that both interfere with herbivores directly and would be exerted indirectly via increasing attractiveness of foraging predators and host-seeking parasitoids (Walling, 2000). Indirect defenses, e.g. attraction of natural enemies to insect pests by emitting plant volatiles, are as well an important mechanism of plants to cope with the infestation by herbivores (Wondafrash et al., 2013). Blends of plant volatiles could be also altered by the indirectly above-below ground interactions, thereby impacting host's cry for help (Kant et al., 2015). Hence, plant-mediated indirect interactions between above ground and below ground pests should preferably be studied in a tri-trophic system. Understanding and manipulating these interactions may result in better pest control, reduce use of pesticides, and contribute to optimizing integrated pest management in agroecosystems.

As observed in the Chapter 3, an interaction was detected among aphids, fungi and nematodes. **The aphid population was resuced by the indirect interactions with powdery mildew fungi and root-knot nematodes, whether the three species of pests attacked**

tomato plants simultaneously or sequentially. In the experiment, this group of the three pests was further used to examine the long-term effects caused by the plant-mediated indirect interactions among above ground and below ground pests on the aphid population dynamic, the biocontrol efficiency by parasitoids, and the yield of tomato fruits. Furthermore, the morphological modulation (wing dimorphism) and spatial distribution (both horizontal and vertical) of aphid populations was investigated as well in this study.

2. Materials and methods

2.1. Study organisms

Tomato plants, *Solanum lycopersicum* L., (cultivar ‘San Marzano Nano’), were grown in a climate controlled greenhouse (23.8±0.3°C in daytime, 19.0±0.2°C at night, RH: 82±1%, irradiance: 880±61 W/m²). They were planted in the substrate combination which contains soil and perlites and supplied with a standard nutrient solution (pH=6, electrical conductivity (EC) =1.6 mS/cm, ion concentration: 620 mg/L NO₃⁻, 11 mg/L NH₄⁺, 170 mg/L H₂PO₄⁻, 115 mg/L SO₄²⁻, 180mg/L K⁺, 212 mg/L Ca²⁺, 12 mg/L Mg²⁺).

The *M. incognita* nematode isolate used in this study was from the collection in INRA, Sophia Antipolis, France. Infective second-stage juveniles (J2) were recovered from infested tomato plants which grew under greenhouse conditions (Neveu et al, 2003). The *O. neolycopersici* isolate came from tomato plants in INRA, Avignon, France. Spores suspensions were prepared by adequately blending the fully-infested tomato leaves with distilled water. The supernatant was collected for the subsequent quantification of spore concentration which was measured with a Mallasez® cell and adjusted to a final concentration of 10⁵ spores/mL. The colony of aphids, *M. euphorbiae*, was reared on the caged tomato plants at INRA, Sophia Antipolis, France. The parasitoid, *Aphidius ervi*, was provided by the Koppert Biological Systems.

2.2. Experimental design

Four treatments were set up to study the influence of above-below ground interactions on the aphid population dynamic and its biocontrol by parasitoids, using a 2 × 2 factorial design (Table 1). Each treatment was isolated in a nylon mesh which constructed tunnels (height: 2m, width: 1m, length: 5m) and each treatment was replicated four times in a climate controlled greenhouse (the same condition as described above). Tomato seedlings were planted in plastic

pots (height: 13cm, diameter: 16cm) and each tunnel had two rows of 8 tomato plants (Fig.1). The infestation of nematodes, powdery mildew and aphids, and the aphid population monitoring were followed the scenario as shown in Figure 2. Two hundred of J2 nematodes and 10 μ L powdery mildew fungi with a concentration of 10^5 spores/mL were respectively inoculated on all the tomato plants in treatment C and D. Five 3rd-instar apterous aphids per plant were introduced on the four plants in the center location of each tunnel (Fig. 1). One week after aphid infestation on tomato plants, the monitoring of aphid population started and it was conducted every week for 8 weeks. The 16 tomato plants in each tunnel were numbered (Fig. 3). The number of aphid individuals on the plant with odd number were monitored in the odd weeks after the initial aphid release and that on the plants with even number were as well recorded in the even weeks. The number of wingless and winged aphids were both recorded for all the sampling plants. On the location where aphids were initially released, plants were equally divided by 3 parts (up, middle and down parts) based on the number of stem nodes. The two aphid parameters (the number of wingless and winged aphids) in the top, middle and down parts of the aphid release plant were respectively recorded. Additionally, after the 5th monitoring of aphid population, 16 pairs of parasitoids after mating were released in the treatment B and D (Table 1) and the number of mummies on tomato plants was as well recorded from the next measurements. After the last monitoring of aphid population, the yield of tomato fruit and the ratio of ripe fruit was also measured.

Table 1. Treatments used in the experiment

| Treatment | Nematode | Fungus | Aphid | Parasitoid |
|-----------|----------|--------|-------|------------|
| A | - | - | + | - |
| B | - | - | + | + |
| C | + | + | + | - |
| D | + | + | + | + |

+ indicated that the organism was present in the treatment; - indicated that the organism was absent in the treatment.

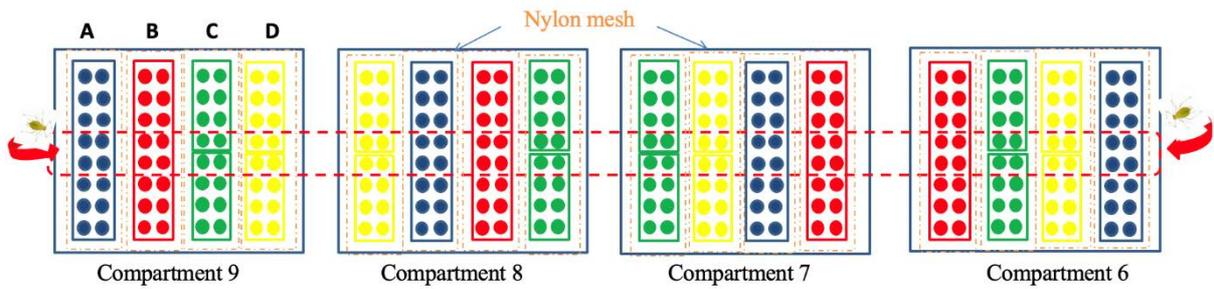


Figure 1. Experimental design of the study. Four compartments were used in the experiment and each compartment contained 4 treatments. Each color represented one treatments (it represented **the whole treatment** in Fig. 5a and Fig. 6a). Different colors indicated different treatments. The initial aphids were released **on the four plants** (it represented **aphid release location** in Fig. 5b and Fig. 6b) in the center location of each tunnel as marked by the red dashed line.

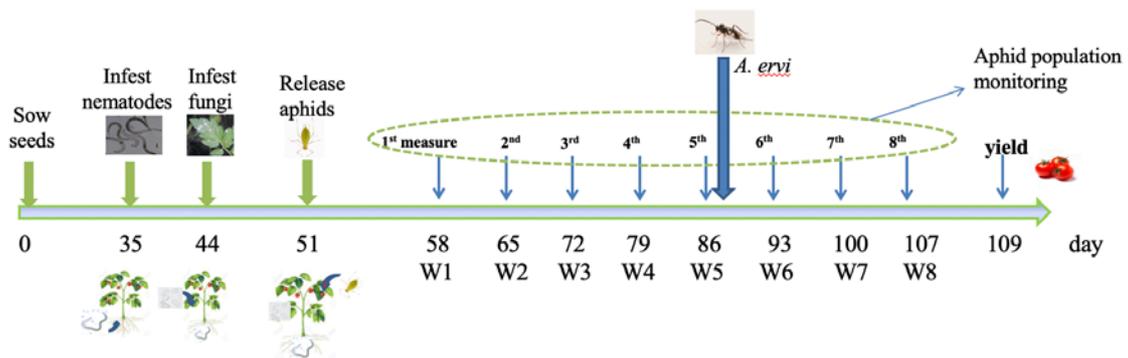


Figure 2. The schedule of organism release and aphid population monitoring during the experiment. Aphid population were monitored every week after the aphid initial release and eight times of monitoring were conducted.

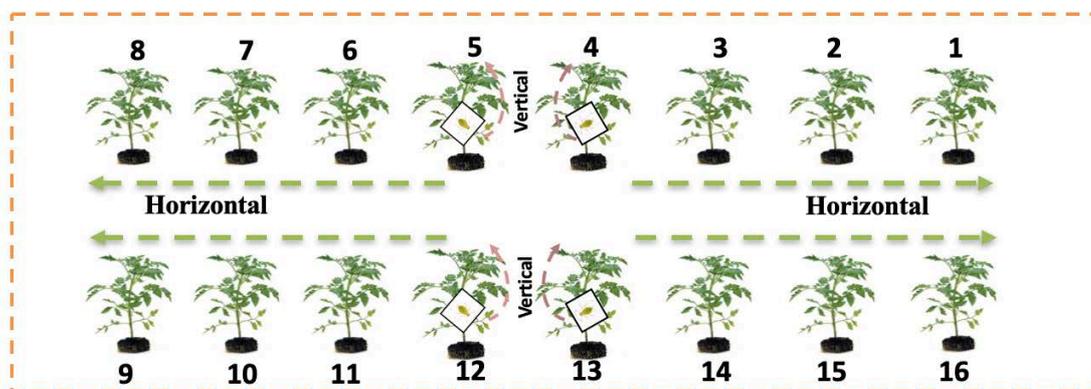


Figure 3. Monitoring methods for the aphid population dynamic. The number of wingless and winged aphids both on the initial release plants and on the non-release plants were measured for the horizontal distribution of aphids in the tunnels. On the initial release plants, the two aphid parameters at different part (up, middle or down part) of plants were as well observed for aphid vertical distribution.

2.3. Data analysis

All the data were analyzed by the Kolmogorov-Smirnov test for their normality and the square root was carried out if it is needed. The significance of the survival of aphid nymphs was analyzed with the univariate analysis in a linear model followed by a Tukey's HSD test for multiple comparisons. The impact of above-below ground interactions (ABI) and the natural enemy (NE) on dynamics of aphid population, proportion of winged aphids and aphid spatial distribution was analyzed by repeated measures with the General Linear Models. The data were divided into four periods, i.e. the initial period of aphid dispersal (1-3 weeks), the period before parasitoid release (1-5 weeks as the short term), the long term (1-8 weeks), and the periods after parasitoids release (6-8 weeks) to examine the effects of ABI and/or NE on aphid populations, according to the dispersal speed of aphids and release time of natural enemies. The effects of ABI on the parasitism rate of parasitoids were as well assessed on the 6-8 week. Two-way ANOVA (analysis of variance) in a General Linear Model was performed to analyze the impact of ABI and NE on the yield and the ripe ratio of tomato fruits. Thereafter, the univariate analysis was carried out and a Tukey's HSD test for multiple comparisons was processed to compare the difference among all the treatments. All the statistical analyses were performed with the software of SPSS 25.0 (SPSS Inc, Chicago, USA).

3. Results

3.1. Impact on the survival of aphid nymphs

In the first week after initial infestation of aphids, the nymph survival of aphids on tomato plants jointly attacked by nematodes and powdery mildew fungi was dramatically lower than that on the tomato plant solely infested by aphids ($F_{3,60} = 9.382$; $P < 0.001$; Fig.4).

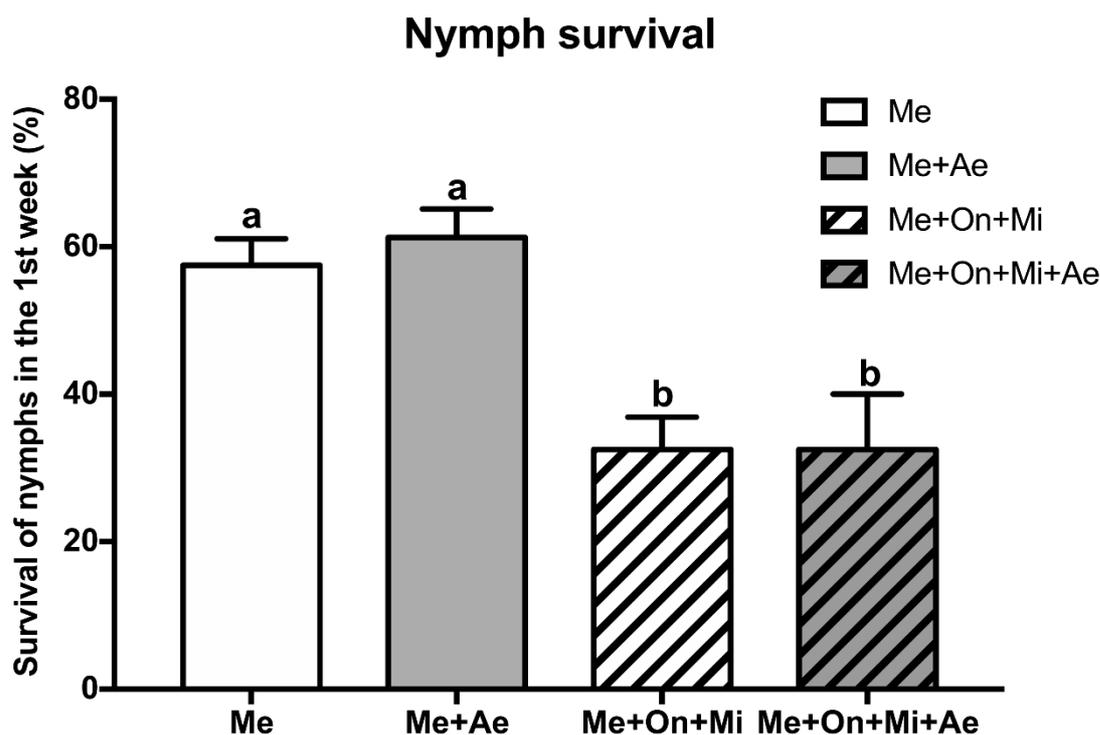


Figure 4. Survival of released aphid nymphs of each treatment in the 1st week.

Me: *M. euphorbiae*; On: *O. neolycoopersici*; Mi: *M. incognita*; Ae: *Aphidius ervi*

3.2. Impact on aphid population dynamics

The statistical results on aphid population dynamics were summarized in Table 2. The number of aphid individuals both in the whole treatment (Fig. 5a) and at the release location (Fig. 5b) kept growing during the 1-7 weeks, but it decreased in the 8th week at the release location.

In the whole treatment, the above-below ground interactions (ABI) among pests had negative significant influences on aphid population during all periods, i.e. 1-3 weeks (initial period of aphid dispersal), 1-5 weeks (short term), and 1-8 weeks (long term). During the 6-8 weeks, i.e. after parasitoid release, the aphid population was negatively affected by both ABI and natural enemies (NE). However, the interaction between the two factors (ABI and NE) had no impact on aphid population density (Table 2; Fig. 5a).

At the initially aphid-release location, the ABI significantly decreased the aphid population during 1-3 weeks and 1-8 weeks. Nevertheless, aphid population was not affected by ABI during 1-5 weeks. In addition, after the release of parasitoids, only ABI had negative effects on aphid population. The NE and the interactions (ABI \times NE) between the two factors had no impact on aphid population during 6-8 weeks (Table 2; Fig. 5b).

Table 2. Statistics from the repeated measures in the General Linear Models used to analyze the impact of above-below ground interactions (ABI) and natural enemies (NE) on dynamics of *M. euphorbiae* population

| Parameters | Location | Time period (weeks) | Factor | <i>df</i> | <i>F</i> | <i>P</i> |
|------------------|------------------------|---------------------|----------|-----------|----------|----------|
| Aphid population | The whole treatment | 1-8 | ABI | 1 | 12.291 | 0.003* |
| | | 1-3 | ABI | 1 | 34.122 | <0.001* |
| | | 1-5 | ABI | 1 | 4.619 | 0.05* |
| | | | ABI | 1 | 20.474 | 0.001* |
| | | 6-8 | NE | 1 | 8.112 | 0.015* |
| | | | ABI × NE | 1 | 0.604 | 0.452 |
| | Aphid release location | 1-8 | ABI | 1 | 22.132 | <0.001* |
| | | 1-3 | ABI | 1 | 45.209 | <0.001* |
| | | 1-5 | ABI | 1 | 4.157 | 0.061 |
| | | | ABI | 1 | 23.05 | <0.001* |
| | | 6-8 | NE | 1 | 3.825 | 0.074 |
| | | | ABI × NE | 1 | 1.831 | 0.201 |

P value followed by asterisk (*) indicates that the factor has the significantly impact on the aphid population dynamics at $P \leq 0.05$.

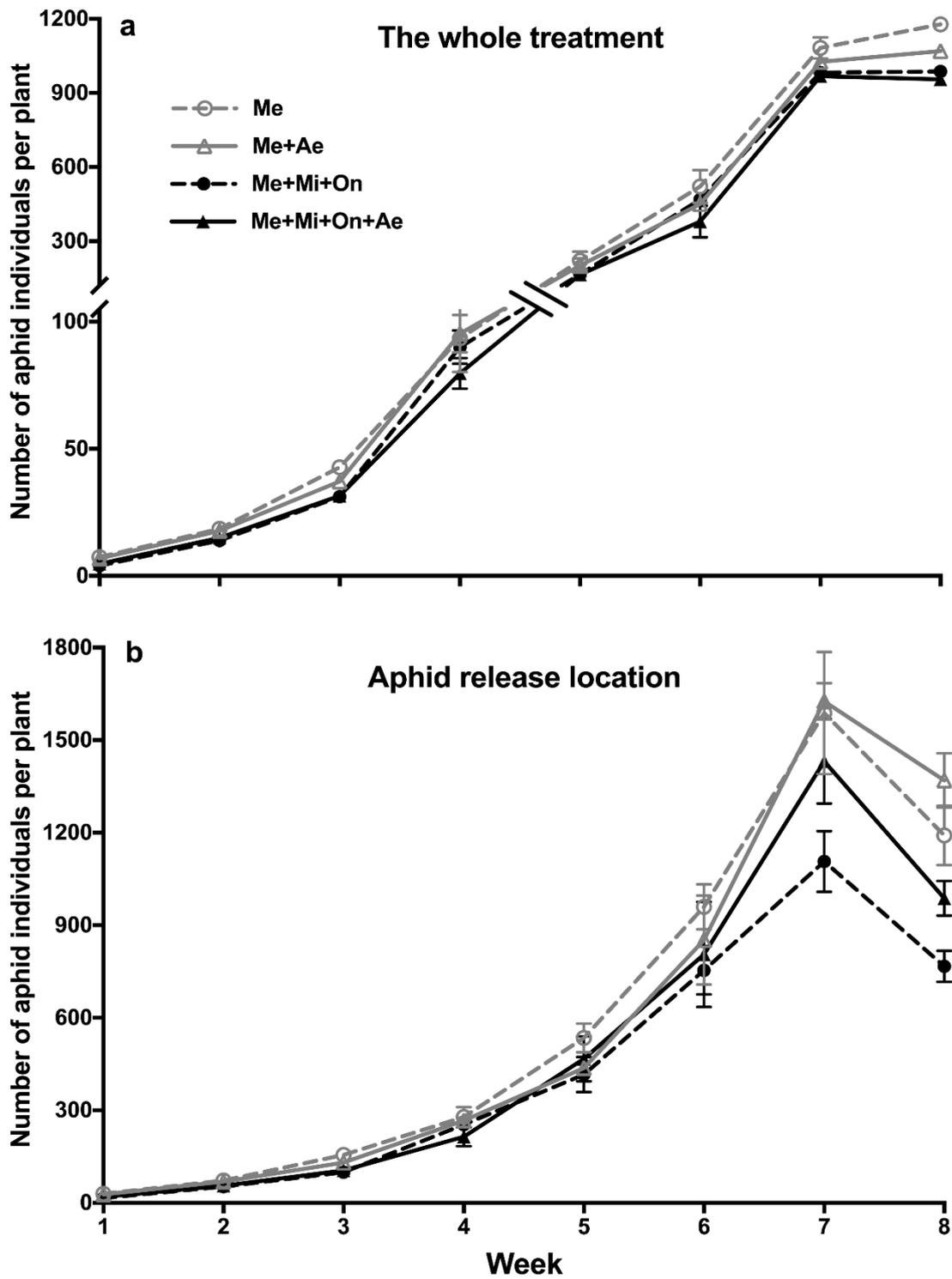


Figure 5. The aphid population dynamics in each treatment.

Me: *M. euphorbiae*; On: *O. neolycopersici*; Mi: *M. incognita*; Ae: *Aphidius ervi*

The whole treatment here contained all the plants (16 plants) in each treatment; aphid release location indicated the central 4 plants where aphids were initially released as shown in Fig. 3.

3.3. Impact on dynamics of alate ratios

The statistical results on dynamics of the percentage of winged aphids were summarized in Table 3. The ratio of alates in aphid population fluctuated at the aphid-release location but it has an increasing trend in the whole treatment (Fig. 6). The ABI significantly increased the percentage of winged aphids on the plants not only in the whole treatment and at the initially aphid-release location during all the four periods. During the 6-8 weeks, the proportion of winged aphids was positive impacted by both ABI and NE in the whole treatment but no effect was detected on the ratio of dispersal morphs by their interactions (ABI × NE) (Fig. 6a). Moreover, on the plants of initial aphid release, the percentage of winged aphids was only increased by the ABI after parasitoid release (6-8 weeks) (Fig. 6b).

Table 3. Statistics from the repeated measures in the General Linear Models used to analyze the impact of above-below ground interactions (ABI) and natural enemies (NE) on the dynamic of alate ratio in the *M. euphorbiae* population.

| Parameters | Location | Time period (weeks) | Factor | <i>df</i> | <i>F</i> | <i>P</i> |
|----------------------|------------------------|---------------------|----------|-----------|----------|----------|
| Percentage of alates | The whole treatment | 1-8 | ABI | 1 | 58.906 | <0.001* |
| | | 1-3 | ABI | 1 | 8.956 | 0.010* |
| | | 1-5 | ABI | 1 | 68.905 | <0.001* |
| | | | ABI | 1 | 70.373 | <0.001* |
| | | 6-8 | NE | 1 | 24.484 | <0.001* |
| | | | ABI × NE | 1 | 0.871 | 0.369 |
| | Aphid release location | 1-8 | ABI | 1 | 36.711 | <0.001* |
| | | 1-3 | ABI | 1 | 5.937 | 0.029* |
| | | 1-5 | ABI | 1 | 24.182 | <0.001* |
| | | | ABI | 1 | 6.114 | 0.029* |
| | | 6-8 | NE | 1 | 0.123 | 0.732 |
| | | | ABI × NE | 1 | 0.953 | 0.348 |

P value followed by asterisk (*) indicates that the factor has the significantly impact on the ratio of winged aphids in the *M. euphorbiae* population at $P \leq 0.05$.

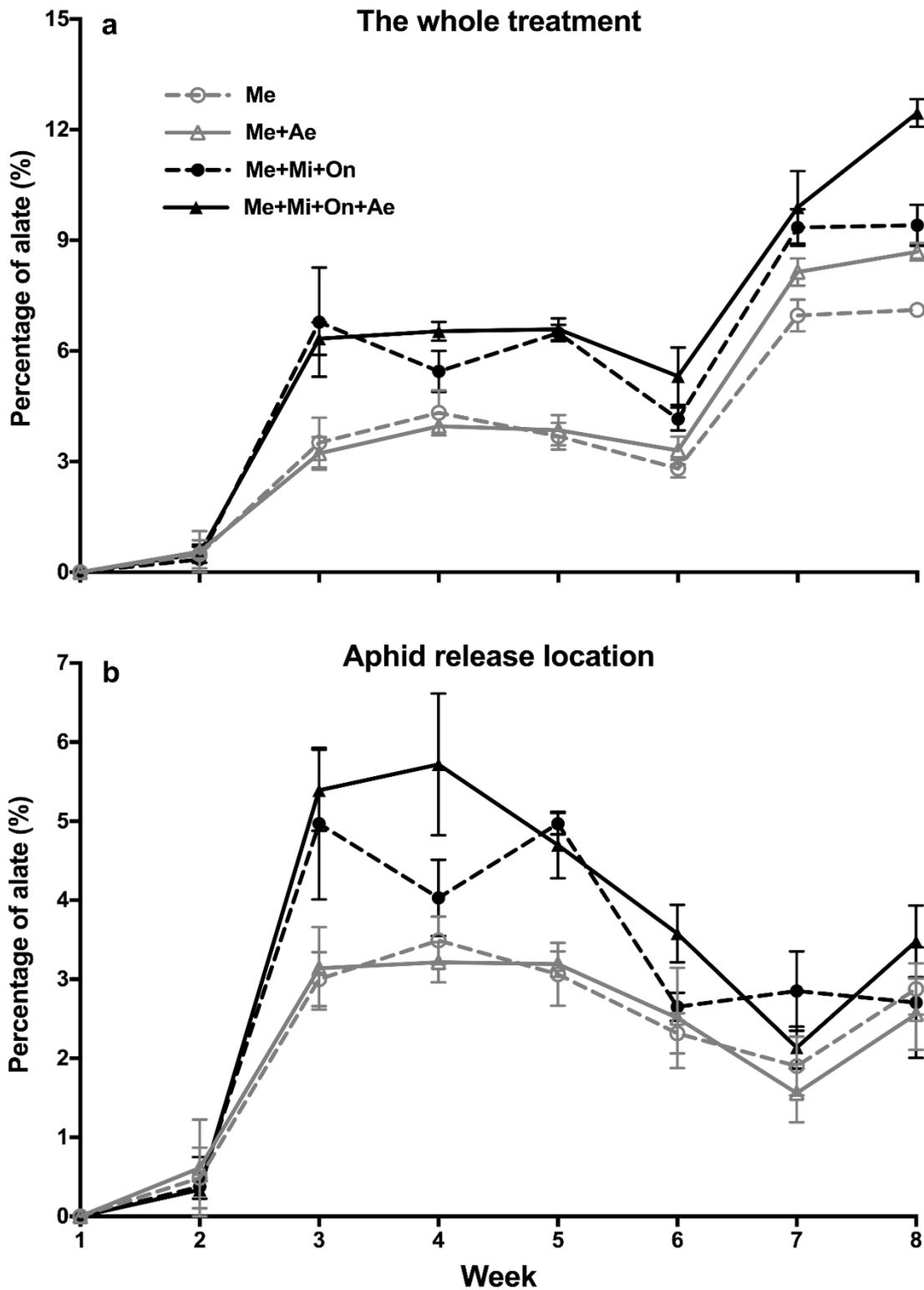


Figure 6. The dynamic of the alate ratio in the *M. euphorbiae* population in each treatment. Me: *M. euphorbiae*; On: *O. neolycopersici*; Mi: *M. incognita*; Ae: *Aphidius ervi*

The whole treatment here contained all the plants (16 plants) in each treatment; aphid release location indicated the central 4 plants where aphids were initially released as shown in Fig. 3.

3.4. Impact on the aphid spatial distribution

The statistical results on the spatial distribution of aphids were summarized in Table 4. In the horizontal direction, all the side plants were detected to be colonized by the winged aphids in the 3rd week. During the migration of dispersal morphs onto the side plants, i.e. during the 1-3 weeks, ABI had a positive effect on the percentage of aphids colonizing on the non-release plants (Fig. 7a). After the release of parasitoids, the proportion of aphids residing on the non-release plants was significantly reduced by NE. However, the horizontal distribution of aphids in the whole treatment was not effected by ABI during other periods, i.e. 1-5 weeks, 6-8 weeks and 1-8 weeks.

Aphids did not arrive at the top part in the aphid-release plant until the 3rd week. Therefore, the statistic values for vertical distribution during 1-3 weeks is not available. The percentage of aphids residing on the top of aphid release plants was significantly increased by the ABI during the 1-5 weeks, 6-8 weeks and 1-8 weeks but the NE and the interactions (ABI × NE) had no influence on vertical distribution of aphids after parasitoid release (Fig. 7b).

Table 4. Statistics from the repeated measures by the General Linear Models used to analyze the impact of above-below ground interactions (ABI) and natural enemies (NE) on dynamic distribution (horizontal and vertical) of *M. euphorbiae*.

| Parameters | Location | Time period (weeks) | Factor | <i>df</i> | <i>F</i> | <i>P</i> |
|----------------------|------------|---------------------|----------|-----------|----------|----------|
| Spatial distribution | Horizontal | 1-8 | ABI | 1 | 1.227 | 0.287 |
| | | 1-3 | ABI | 1 | 4.701 | 0.048* |
| | | 1-5 | ABI | 1 | 0.443 | 0.516 |
| | | | ABI | 1 | 3.199 | 0.099 |
| | | 6-8 | NE | 1 | 5.831 | 0.033* |
| | | | ABI × NE | 1 | 1.506 | 0.243 |
| | Vertical | 1-8 | ABI | 1 | 16.485 | 0.001* |
| | | 1-5 | ABI | 1 | 13.57 | 0.002* |
| | | | ABI | 1 | 5.97 | 0.031* |
| | | 6-8 | NE | 1 | 0.076 | 0.787 |
| | | | ABI × NE | 1 | 0.758 | 0.401 |

P value followed by asterisk (*) indicates that the factor has the significantly effect on aphid spatial distribution at $P \leq 0.05$.

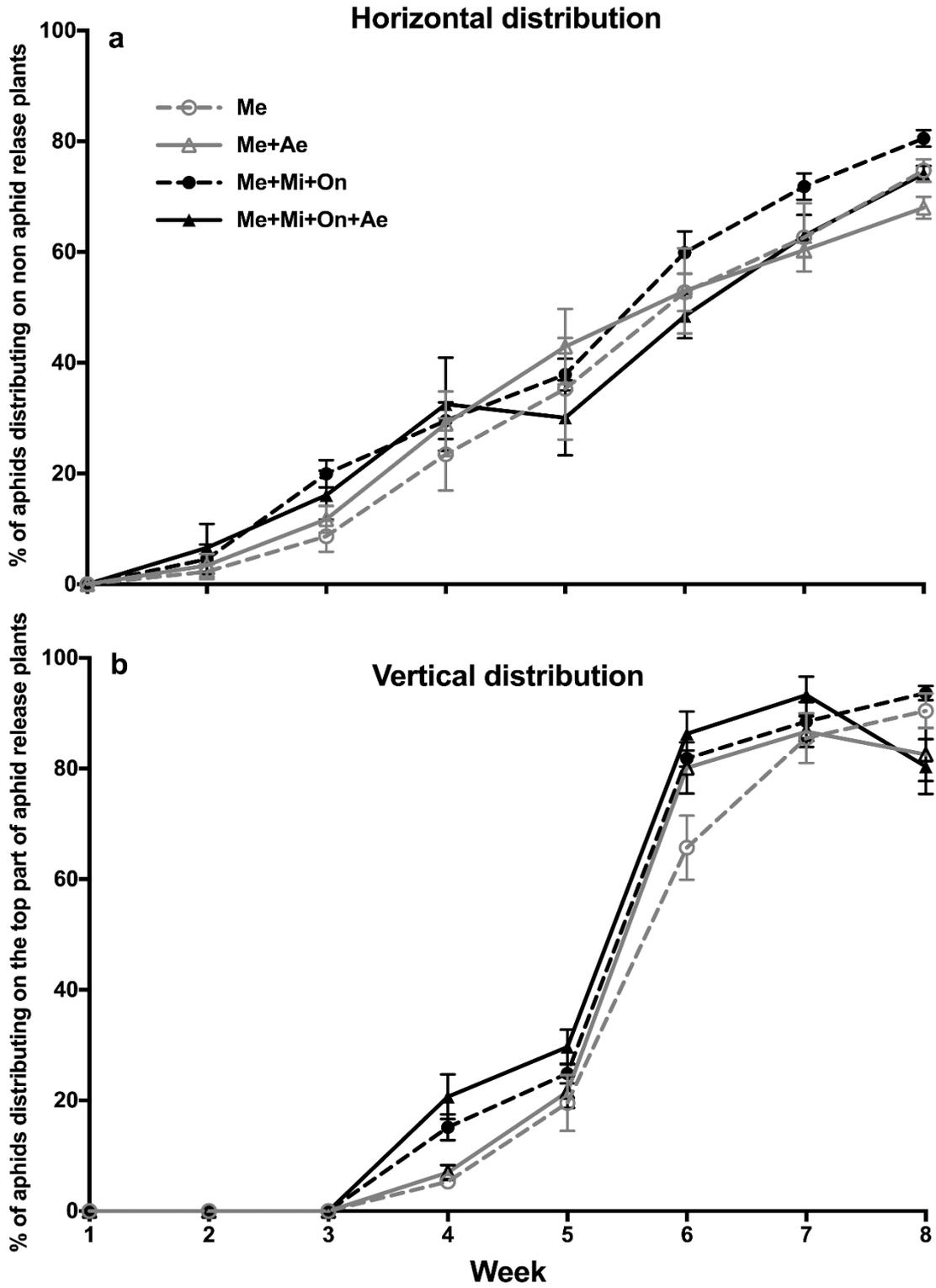


Figure 7. The dynamic spatial distribution of aphids in each treatment.

Me: *M. euphorbiae*; On: *O. neolycopersici*; Mi: *M. incognita*; Ae: *Aphidius ervi*

3.5. Impact on biocontrol

The parasitism rate of *A. ervi* to *M. euphorbiae* was significantly decreased by the above-below ground interactions after the release of parasitoids, i.e. during the 6-8 weeks.

Table 5. Statistics from the repeated measures by the General Linear Models used to analyze the impact of above-below ground interactions (ABI) on the parasitism rate of *A. ervi*.

| Parameters | Location | Time period (weeks) | Factor | <i>df</i> | <i>F</i> | <i>P</i> |
|------------------------|---------------------|---------------------|--------|-----------|----------|----------|
| Parasitism rate of NEs | The whole treatment | 6-8 | ABI | 1 | 13.919 | 0.01* |

P value followed by asterisk (*) signifies that the factor (ABI) has the significantly effect on parasitism rate of NEs at $P \leq 0.05$.

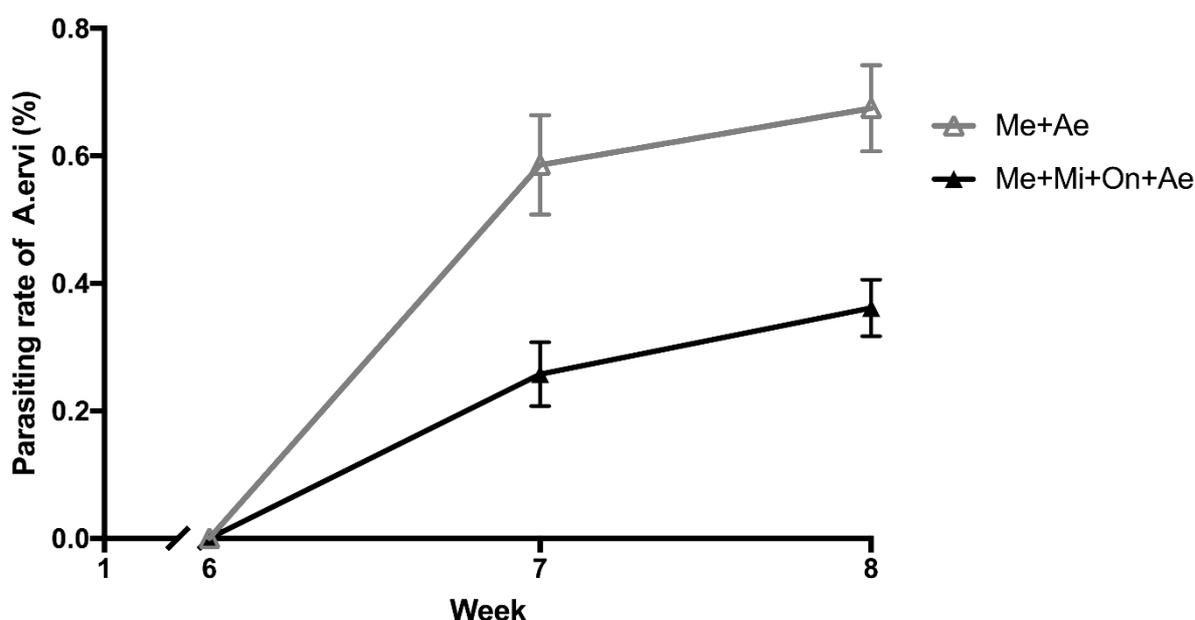


Figure 8. The dynamic of the parasitism rate of *A. ervi* to *M. euphorbiae*.

Me: *M. euphorbiae*; On: *O. neolyopersici*; Mi: *M. incognita*; Ae: *Aphidius ervi*

3.6. Impact on tomato yields

The ABI had significantly negative effects on the weight of tomato fruits but it did not impact the ratio of ripe fruits. In addition, both the tomato yields and ratio of ripe fruits were not affected by the NE and the interaction (ABI \times NE) of the two factors (Table 6; Fig. 9).

Table 6. Statistics of two-factor analysis on the effects of ABI and NE on tomato yields and the ratio of ripe fruits.

| Tomato fruit | Factor | df | F | P |
|--------------|----------|----|-------|--------|
| Yield | ABI | 1 | 7.27 | 0.019* |
| | NE | 1 | 2.22 | 0.162 |
| | ABI × NE | 1 | 0.177 | 0.681 |
| Ripe ratio | ABI | 1 | 0.641 | 0.439 |
| | NE | 1 | 0.866 | 0.37 |
| | ABI × NE | 1 | 0.051 | 0.825 |

P value followed by asterisk (*) signifies that the factor has the significantly effect on the yields of tomato fruit at $P \leq 0.05$.

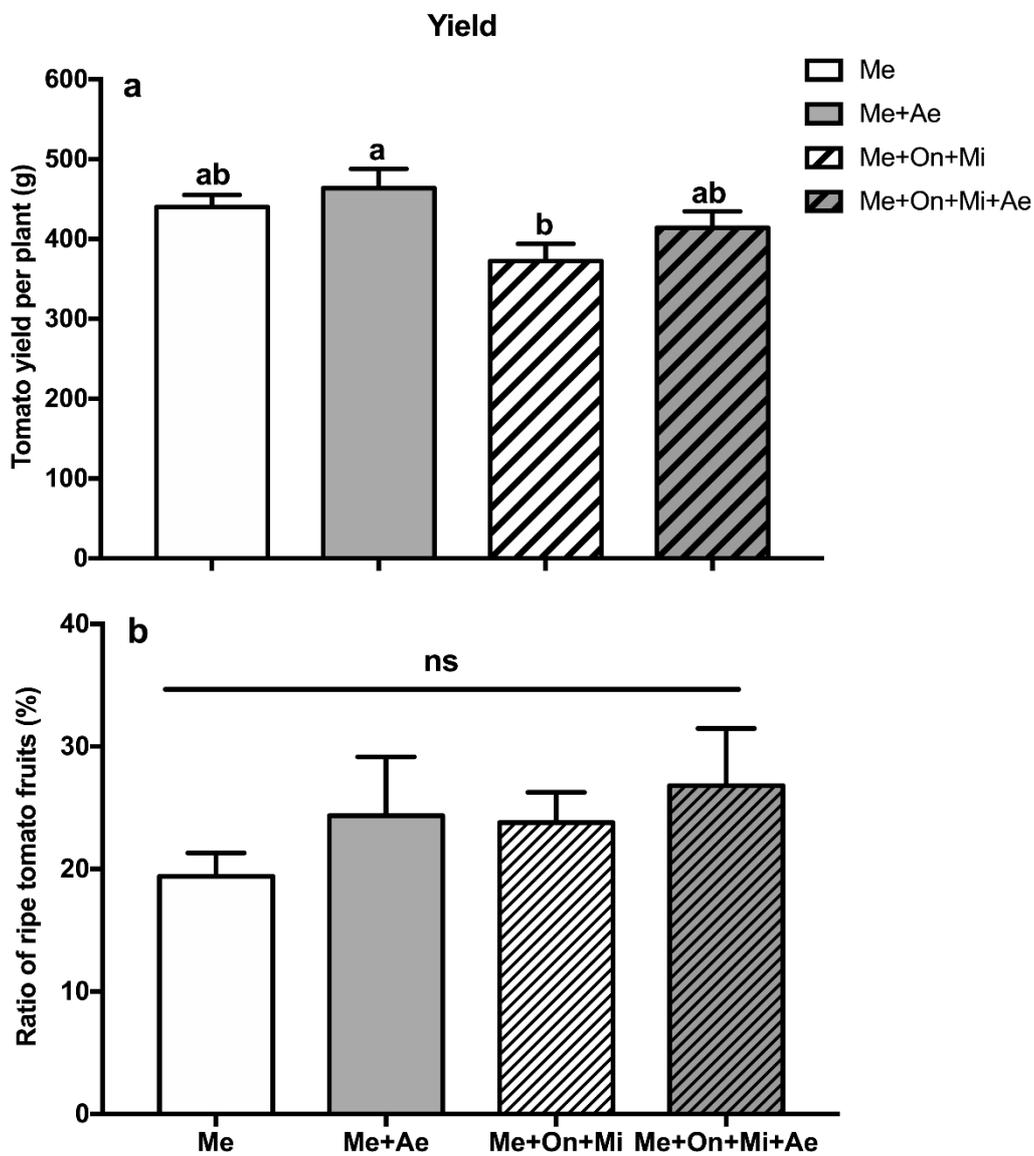


Figure 9. The tomato yields and the ratio of ripe fruits in different treatments.

Me: *M. euphorbiae*; On: *O. neolyopersici*; Mi: *M. incognita*; Ae: *Aphidius ervi*

4. Discussion

Plants employed both constitutive defenses and induced defenses to cope with the attack by pests (War et al., 2012). Plants can not only use hairs, trichomes, thorns or spines as the mechanical protection on plant surface but also produce toxic chemical compounds, such as polyphenols, alkaloids, terpenoids or defensive lectins to protect themselves from biotic stress (Hanley et al., 2007). In tomato, it has been known that α -tomatine and tomatidine were produced to defend against herbivorous insects and plant pathogens (Chowański et al., 2016). Alterations in plant defensive constituents due to pest attack, in turn, negatively impact herbivorous performances. In our results, feeding on the infestation-free tomato plants caused around 40% nymph mortality of aphids (Fig. 4). The decreased survival of nymph aphids in all treatments may be linked to the plant defenses (Jaouannet et al., 2014). In addition, as plant defenses have been triggered by nematodes and fungi early, the defensive responses to the subsequent aphids will be more intense compared to those plants which were attacked solely by aphids (War et al., 2012). More toxic secondary metabolites would be accumulated on the host plant induced by nematodes and powdery mildew fungi (Bleve-Zacheo et al., 2007). This could be the main reason that resulted in the lower survival in aphid nymphs involved in the above-below ground interactions on tomato plants. The adverse influence on aphid individuals, i.e. the higher mortality of aphids on the plants pre-infested by nematodes and fungi, may lead to a further negative effect on the aphid population dynamic.

Wing polyphenism is a common phenomenon displayed in many insect species (Müller et al., 2001). Within aphids, wing dimorphism is also vital for the life cycle and the winged morphs (alates) appear in response to the environmental changes so as to benefit migration to new fresh resources (Braendle et al., 2006; Brisson, 2010; Dixon, 1998). Although the alates are good at migration, the wingless individuals (apterous aphids) have a faster developmental time, larger body size and higher fertility as it is costly for aphid to produce the winged morphs (Braendle et al., 2006). This could be another reason to explain how the above-below ground interactions decreased aphid population, i.e. the lower aphid population density was achieved by induced more winged morphs with lower fertility in the aphid colony on the tomato plants pre-infested by nematodes and fungi. The possible cues of wing induction include crowding/density (tactile simulation), host quality (nutrition), pressure of natural enemies, and temperature, and so on (Kunert et al., 2005; Müller et al., 2001). Hence, the proportion of winged aphids increase gradually with the elevation of aphid density over time in all treatments due to the more crowding and depleted host nutrition (Fig. 5a; 6a). Moreover, in my results, both the above-below ground indirect interactions and parasitoids facilitate the induction of

dispersal morphs in aphid colonies. The increasing induction of winged morphs on the plants jointly infested by nematodes and fungi may be linked to the alteration of plant physiology, e.g. concentration of amino acids. It has been documented that the concentration of nitrogen in leaves was significantly reduced by pre-infestation of nematodes *M. incognita* on tomato roots (Guo and Ge, 2017). In addition, plant pathogens that were interacted with aphids on the same host have been as well reported to benefit the formation of winged individuals (Müller et al., 2001). For instance, aphid species of *Sitobion avenae* and *Rhopalosiphum padi* produced more winged offspring when they fed on virus-infested barley than those on healthy plants (Gildow, 1980). On the plants where aphids were initially released, the pre-infestation by nematodes and fungi caused significantly lower aphid density (Fig. 5b). However, the indirect interactions among the above-below ground pests resulted in severe movement of aphids towards the upper parts for the better nutrition within the tomato plant (Fig. 7b). The stronger mobility of aphids resulted from the plant-mediated indirect interactions may cause ‘pseudo crowding’ effect, thereby leading to more winged individuals in aphid colonies. In the third week, the winged individuals arrived on the side plants in all treatments. During the 1-3 weeks, the larger horizontal distribution of aphids on non-aphid release plants in the treatments involving above-belowground interactions relies mainly on the migration of winged aphid individuals that were induced more by the pre-infestation of nematodes and fungi (Fig. 6a; 7a). Furthermore, our results are consistent with the Sloggett and Weisser’s study on the wing induction by *A. ervi*, i.e. the pea aphid *Acyrtosiphon pisum* produced a significantly higher proportion of winged aphids when it was exposed to the parasitoids (Sloggett and Weisser, 2002). Because natural enemies can cause aphids to be restless, they move a lot to avoid to be attacked by parasitoids (Braendle et al., 2006; Kunert et al., 2005). They are, therefore, more likely to encounter each other during the movement, wing formation may be induced by the crowding cue (Braendle et al., 2006; Kunert et al., 2005). Aphids can succeed in escaping from the attack of the parasitoids by kicking at them and therefore the physical contact with parasitoids may also lead to wing induction in aphid individuals (Müller et al., 2001).

Plants would make a trade-off between the plant defense and the growth and they prefer to allotting the photosynthate to the much-needed tissues, such as to the plant head with apical meristems for plant growth, in addition to the costly expenditure on induced defense caused by pre-infestation by nematodes and fungi, therefore driving aphid up for fresher nutrients (Kaplan et al., 2008b; Tao and Hunter, 2013). In the first three weeks after aphid release, the plant resource was rich for the small quantity of aphid population. Therefore, the vertical spread was very less and even no aphid colonized the top part of plant for resource exploitation before the

3rd week. However, as the host resources were more consumed on tomato plants attacked by the multiple pests (nematodes, fungi and aphids) than those on plant infested by aphids alone, aphids, subjected to the above-below ground interactions, scrambled more to colonize better resources at the top part over time.

The suppressed population of aphids on tomato plants pre-infested by nematodes and fungi in our results is consistent with another study on the above-below ground interactions among three pests involving the nematode *Heterodera glycines*, the plant fungus *Cadophora gregata* and the aphid *A. glycines* on soybeans (McCarville et al., 2012). This may be supported by the induced defense hypothesis, which indicates that the pre-infestation by the root pest (insect or pathogen) boosts the foliar defenses against future damage by inducing or elevating a systemic defense in leaves (Annapurna et al. 2013; Jung et al. 2012; Weller et al. 2012). For instance, the SA-dependent defensive signaling against the sap-feeder whiteflies on tomato leaves was enhanced by the previous infection of *M. incognita* on tomato roots, leading to the population suppression of whiteflies on tomato plants (Guo and Ge, 2017). Additionally, the interactions among distinct phytohormonal signaling pathways triggered by different feeding guilds of pests may be additive, synergistic or antagonistic, which also shape the outcome of plant-mediated indirect interaction among multiple species of pests (Koornneef and Pieterse, 2008). Hence, when plants are attacked by more than one pest species, the palette of plant defenses induced together will determine their mutual interactions and the effects on their natural enemies.

Plants can emit some plant organic volatiles to attract the natural enemies of herbivorous insects to reduce the risk of attack by their biotic consumers. However, more than 250 insect species, including aphids, have been reported that they are able to sequester plant toxic metabolites, e.g. alkaloid, to tolerate plant induced defenses (Opitz and Müller, 2009). Moreover, glucosinolates, as the specific secondary metabolites produced by the *Brassica* plants to defend against pests, have been documented to impact the performance of a third trophic level (Hopkins et al., 2009; Kos et al., 2012; Poelman et al., 2008a). Parasitoids could be interfered by an indirect ingestion of such plant toxic compounds due to the trophic cascade effects. Therefore, when aphids are involved in a complex indirect interaction (above-below ground indirect interactions among nematodes, fungi and herbivores) on one host, they have potential to contact more diverse plant defensive metabolites which induced by other pests, especially arriving on the host earlier than aphids. This could be one of the factors resulting in the reduced parasitism efficacy of *A. ervi* to *M. euphorbiae* on tomato plants pre-infested by *M. incognita* and *O. neolycopersici*. Ultimately, fruit yields were negatively affected (Table 6,

Fig. 9a) by the indirect interaction among above ground and below ground pests, via the reduced control efficacy of parasitoids and lessening the shoot fresh weight and photosynthetic rate of leaves on tomato plants (Gue and Ge, 2017).

To conclude, the remarkably reduced survival of aphid nymphs was caused by the indirectly above-below ground interactions, which may be the one of the reasons resulting in the lower aphid population. In addition, the above-below ground interactions promoted the differentiation of winged morphs not only in the short term but also in the long term, which could be the other cause to slow down aphid population. Moreover, the facilitation to horizontal spread of aphids may benefit from the increasing ratio of alates in the aphid population during the first three weeks. The above-below ground interactions activated vertical migration of aphids to the top part of tomato plant for the better conditions of survival and nutrients, which in turn, facilitate the formation of dispersal morphs of aphids, via increasing the mobility of aphids. Furthermore, parasitoids increased the formation of winged aphids and also slow down the aphid population. However, the efficiency of parasitoids against aphids was reduced by the above-below ground interactions among aphids, fungi and nematodes and eventually the yields of tomato fruits were significantly reduced by the combined infestation of above-below ground pests due to the depleted nutrition in the host.

CHAPTER 5. THE UNDERLYING CHEMICAL MECHANISMS OF PLANT-MEDIATED INDIRECT INTERACTIONS

1. Introduction

To date, the genome, transcriptome, and proteome are presented in terms of a ‘signal flow’ and the metabolome is considered as the ‘result’ (Tsugawa, 2018). Additionally, many studies have documented that metabolites are deeply involved in the physiological functions and homeostasis of living organisms (Li et al., 2014; Rooks and Garrett, 2016; Shibata et al., 2017). Metabolome is the complete set of small-molecule chemicals found within a biological sample that can be cells, an organ, a tissue, a tissue extract, or an entire organism (Oliver et al., 1998).

Many aspects of the way plants protect themselves against pathogen attack, or react upon such an attack, are realized by metabolites (Gunnaiah et al., 2012; Ibáñez et al., 2010). The ambitious aim of metabolomics, namely the identification and annotation of the entire cellular metabolome, poses a considerable challenge due to the high diversity of the metabolites in the cell. Nevertheless, it is developed rapidly as machines and platforms are becoming more accurate and sensitive, and public metabolite databases enable improved annotation (Tenenboim and Brotman, 2016). Recent advances in analytical methods and data analysis have resulted in improved sensitivity, accuracy, and capacity, and make it feasible to analyze hundreds to thousands of compounds within one sample (Aliferis and Jabaji, 2012; Sade et al., 2014). Metabolomics have become a popular tool that is now widely used to elucidate the underlying mechanisms of plant biotic interactions, including plant defenses, induced resistance, multiple biotic and abiotic stresses, allelopathy, and more (Erb et al., 2015; Parker et al., 2009; Routaboul et al., 2012; Tzin et al., 2015; Weldegergis et al., 2015).

Depending on the classes of compounds that are main interested, the different extraction methods and analytical platforms (one or more) are chosen (Hall, 2006). When standards are available, numerous metabolites that belong to different metabolic pathways can be simultaneously detected and even quantified with the selected platform. Moreover, untargeted metabolomics approaches, including unbiased data processing, can reveal a comprehensive view of the relative levels of hundreds of metabolites in crude tomato fruit extracts (De Vos et al., 2011). However, the majority of metabolites usually detectable in tomato extracts are not yet structurally characterized. Such untargeted approaches can provide valuable information on the global metabolomics differences and similarities between different samples, in addition to the identification and quantification of the metabolites (Iijima et al., 2008; Mintz-Oron et al., 2008; Moco et al., 2006, 2008).

It may help to further study the constitutive and inducible defenses by focusing on the modulations of plant biochemical or physiological traits that mediate the indirect interactions among multiple plant attackers. **In our study, the metabolomics of tomato plants attacked by multiple species of pests were analyzed with the UHPLC-HRMS. In addition, blends of constitutive and induced volatiles of tomato plants were analyzed with GC-MS as well.**

2. Non-volatile metabolomics analyses of tomato roots under plant-mediated indirect interactions

2.1. Materials and methods

2.1.1. Sample preparation

This sampling followed the same experiment in Article 2 and all the pest infestation were as shown in Fig. 1. After the life-history traits of pests (nematodes, fungi, aphids and caterpillars) were measured (done in Article 2), all the local roots and leaves with pest infestation were collected at DAS 61 and stored in -80°C . Because the metabolomics analyses on leaves are on-going, here, only the results on roots were shown. There were 8 treatments (Treatment **D, G, I, J, L, M, N, O**) and control (**CG**). All the samples were lyophilized (Serail RP2V) for 48 hours to remove moisture. Then, they were ground with a ball mill (Retsch MM301) by shaking (30/s) with 2 metal balls with a diameter of 2cm for 2 minutes. The root powder was transferred to 2 ml Eppendorf tubes and stored in -80°C until the next step of metabolite extraction (*This part of work was conducted in ISA of INRA*).

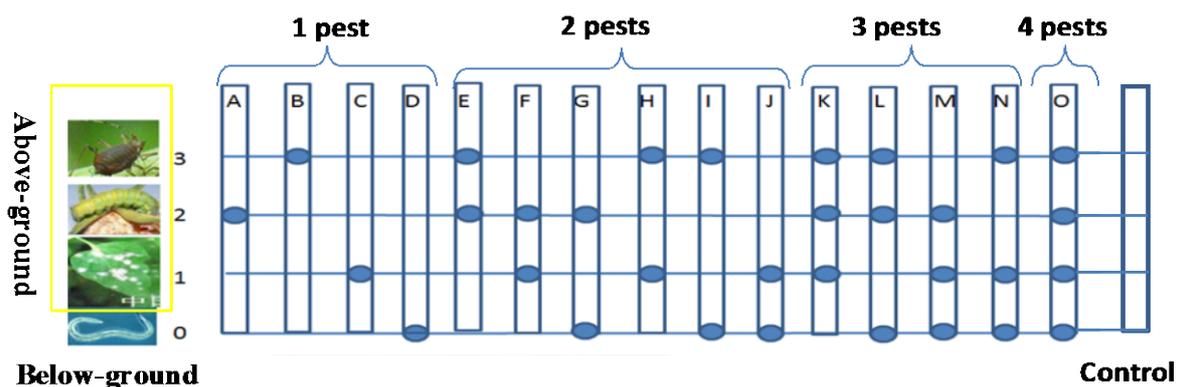


Figure 1. Experimental design of pest infestation in each treatment. Each blue point indicates this species of pest presence on tomatoes.

2.1.2. Sample extraction

The chemical extraction was performed by collaboration with ICOA of Université d'Orléans.

The detailed methods referred to the 'method manuscript' in ANNEX 1

2.1.3. UHPLC-ESI-Q-ToF-HRMS analysis

The UHPLC-ESI-Q-ToF-HRMS analyses were as well performed in ICOA of Université

d'Orléans by a collaboration. The detailed methods referred to the 'method manuscript' in ANNEX 1

2.1.4. Data analysis

Preprocessing of the data were as well conducted by our collaborator in ICOA of Université d'Orléans. The detailed methods referred to the 'method manuscript' in ANNEX 1

Thereafter, the matrices of peaks in each treatments were gotten and I conducted the subsequent data analyses *as follows*:

The matrices of peak intensity include 90 samples and 855 variables imputed by intensity values. The post-hoc analyses among pest-infestation treatments and control was performed by MetaboAnalyst 4.0 and the auto-scaling algorithm was used to normalize the variable intensity. Both univariate (T test) and multivariate analysis (PLS-DA) were used to identify differences among different groups.

2.2. Results

2.2.1. Tomato root metabolomics analyses between control and pest-infested plants

The components (Fig. 2a) explained by the Control (CG) and Treatment (T) was separated from the scores plot of 2D (Fig. 2b) and 3D (Fig. 2c) in PLS-DA. The important features were achieved by the multivariate analysis and the first significant 15 iron fragments were shown in the Figure 3a. And then, a univariate analysis (T test, Fig. 3d) was carried out for the significant molecules in the metabolomics between control plants and pest-infestation plants. Thereafter, the comparison between each treatment and control was also performed the same analysis procedure described above. As a whole, 316 significant molecules were detected in roots between control and all nematode-infestation treatments (Table 1).

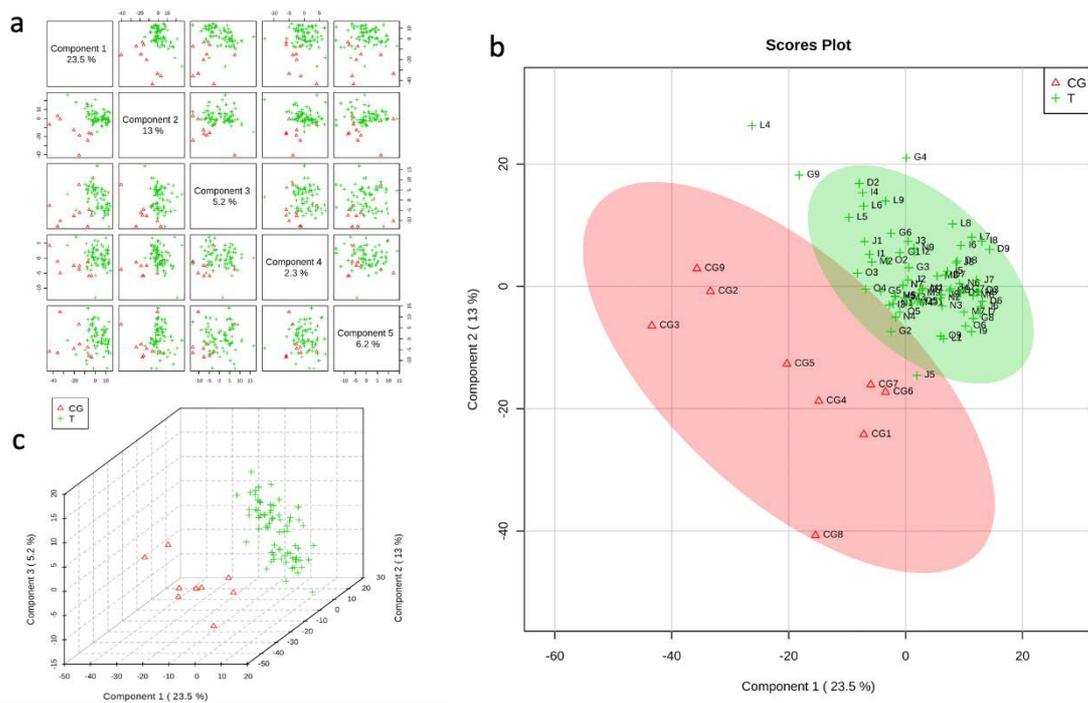


Figure 2. Pairwise (a), 2D (b) and 3D (c) scores plots between the selected components. (a): the explained variance of each component is shown in the corresponding diagonal cell. The components of all nematode-infested treatments (T) are shown in green, and the components of control (CG) are shown in red. (b): the explained variances are shown in brackets. (c): the explained variances are shown in brackets. CG indicates the control roots without any infestation in this plant; T indicates all the treatments involving nematode infestation.

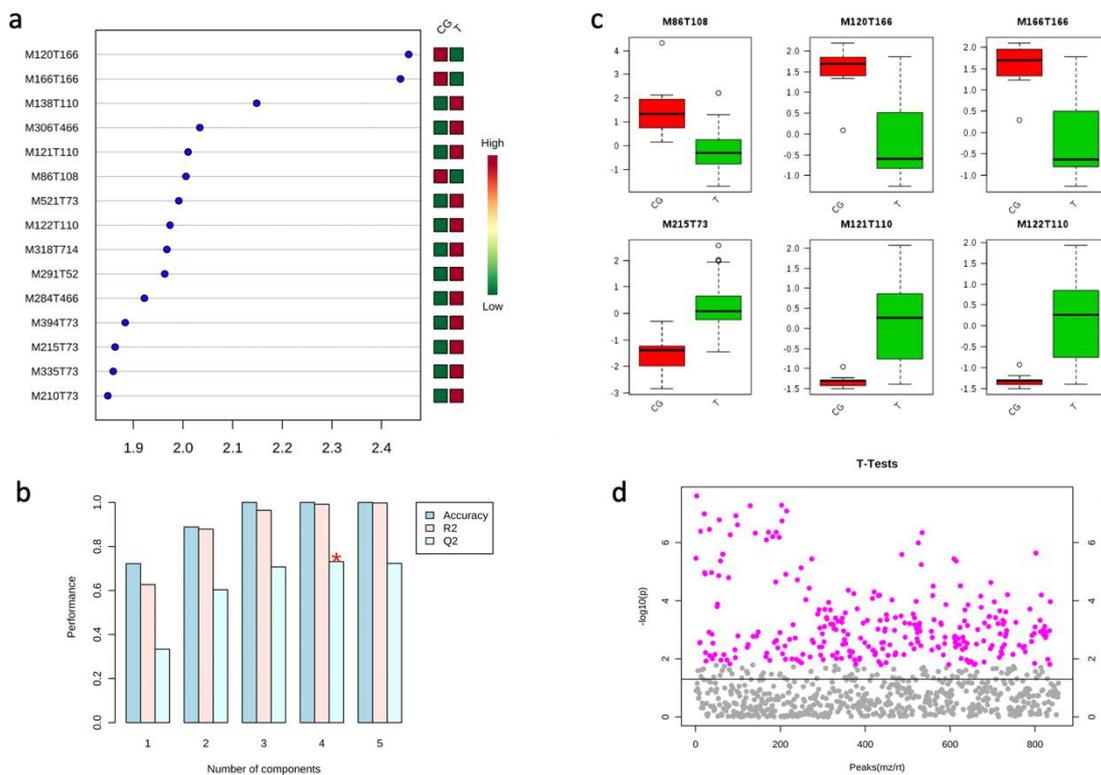


Figure 3. Multivariate (PLS-DA) and univariate analysis (T test) between control and treatments. (a): important features identified by PLS-DA. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in each group under study. (b): PLS-DA classification using different number of components. The red star indicates the best classifier. (c): significantly different features (up-regulated ones, such as M215T73, M121T110 and M122T110; down-regulated such as M86T108, M120T166 and M166T166) between control and all the treatments. (d): important features selected by t-tests with threshold $P < 0.05$. The red circles represent features above the threshold ($P < 0.05$). The P values are transformed by $-\log_{10}$ so that the more significant features (with smaller P values) will be plotted higher on the graph.

CG indicates the control roots without any infestation in this plant; T indicates all the treatments involving nematode infestation.

Table 1. Significant features detected between treatments and control. Me: *M. euphorbiae*; On: *O. neolyopersici*; Mi: *M. incognita*; Ha: *H. armigera*

| Control CG | 1 pest | 2 pests | | | 3 pests | | | 4 pests |
|--|---------|------------|------------|------------|---------------|---------------|---------------|------------------|
| | D Mi | G Mi+Ha | I Mi+Me | J Mi+On | L Mi+Ha+Me | M Mi+Ha+On | N Mi+Me+On | O Mi+Ha+Me+On |
| The number of significant features | 120 | 114 | 120 | 120 | 120 | 120 | 120 | 120 |
| M295T50 | | | down | | | | | |
| M330T50 | | | down | | | | | down |
| M118T51 | | down | | | | | down | down |
| M142T51 | up | up | up | up | up | up | up | up |
| M348T51 | | | | | | | | up |
| M136T52 | | | down | | | down | | |
| M152T52 | | | | | up | | | |
| M278T52 | up | up | | | up | | | |
| M291T52 | up | up | up | up | up | up | up | up |
| M322T52 | up | up | | up | up | up | | |
| M131T53 | | up | | | | | | |
| M140T53 | up | up | up | up | up | up | up | up |
| M154T53 | up | | | up | | | | |
| M323T53 | up | up | | up | up | | | |
| M405T53 | up | up | | | up | up | | |
| M157T54 | up | | | up | up | up | up | |
| M313T54 | | | | | up | | | |
| M179T55 | down | | | | down | | | |
| M190T55 | | | | | | | up | |
| M136T69 | | up | down | | | | down | down |
| M308T70 | up | | up | | | | | up |
| M332T72 | up | | | | | up | | up |
| M111T73 | up | up | up | up | up | up | up | up |
| M129T73 | up | up | up | up | up | up | up | up |
| M139T73 | up | up | up | up | up | up | up | up |
| M147T73 | up | up | up | up | up | up | up | up |
| M157T73 | up | up | up | up | up | up | up | up |
| M175T73 | up | up | up | up | up | up | up | up |
| M189T73 | up | up | up | up | | up | up | up |
| M193T73 | up | up | up | up | up | up | up | up |
| M202T73 | up | up | up | up | up | up | up | up |
| M210T73 | up | up | up | up | up | up | up | up |
| M212T73 | up | up | up | up | up | up | up | up |

| | | | | | | | | |
|----------|------|------|------|------|------|------|------|------|
| M215T73 | up |
| M216T73 | up |
| M220T73 | up |
| M221T73 | up |
| M231T73 | up | up | up | up | | up | | |
| M249T73 | | up | up | up | | up | up | |
| M292T73 | up | up | | | up | | | |
| M310T73 | up | up | up | | up | | | |
| M335T73 | up |
| M394T73 | up |
| M407T73 | up | up | up | up | up | | | |
| M408T73 | up | up | up | | up | | | |
| M423T73 | up |
| M438T73 | up | |
| M439T73 | up | |
| M521T73 | up |
| M123T74 | | up | | | | | | |
| M132T74 | | up | up | up | | up | | |
| M146T74 | | up | up | up | | up | up | |
| M155T74 | up | up | up | up | | up | up | up |
| M237T74 | | up | up | up | up | up | up | |
| M277T74 | up | up | up | up | up | | | up |
| M84T78 | up |
| M142T78 | up |
| M256T78 | up |
| M140T82 | | up | | | | | | up |
| M332T87 | | up | | | | | | up |
| M130T92 | | up | | | | | | up |
| M307T92 | | up | | | | | | |
| M355T92 | | up | | | | | | |
| M113T94 | | | down | | | | | |
| M267T94 | | | down | | | | | |
| M228T100 | up | up | up | | | | | up |
| M244T103 | | up | | | | | | up |
| M86T108 | down |
| M132T108 | | | down | | | down | down | |
| M121T110 | up |
| M122T110 | up |
| M138T110 | up |
| M314T121 | | | | | | down | down | down |
| M136T124 | | up | | up | | up | up | up |
| M268T124 | up | up | | up | up | up | up | up |

| | | | | | | | | |
|----------|------|------|------|------|------|------|------|------|
| M269T124 | up | up | | up | up | up | up | up |
| M136T124 | up | | | | up | | | |
| M152T134 | | | down | | | | | |
| M270T143 | up | | up | up | up | | | |
| M128T144 | | | | up | | | | |
| M172T144 | | | | up | | up | | |
| M144T145 | | | | up | | up | | up |
| M164T145 | | | | | | up | up | up |
| M245T145 | | | | | | up | | |
| M268T145 | | | | | | | | up |
| M141T156 | | | | | | up | | |
| M325T159 | up | up | | | | | | up |
| M120T166 | down |
| M166T166 | down |
| M457T183 | | | up | | | | | up |
| M387T199 | up | up | | | | up | up | up |
| M193T217 | | | | down | | | down | |
| M136T221 | up | up | up | up | | up | | up |
| M146T221 | down | down | | | down | | | |
| M188T221 | down | down | | down | down | | | |
| M189T221 | down | down | | down | down | | | |
| M205T221 | down | down | | down | | | | |
| M298T221 | up | up | | | | | | |
| M242T239 | up |
| M177T250 | | | | | | | | up |
| M319T253 | | | | down | | | | |
| M135T262 | | | up | up | | | | |
| M145T262 | | | up | up | | | | |
| M163T262 | | | up | up | | | | |
| M164T262 | | | up | up | | | | |
| M177T263 | | | | | down | | | |
| M223T263 | | | | | down | | | |
| M227T262 | | | up | up | | | | |
| M236T262 | | | up | up | | | | |
| M355T262 | | | up | | | | | |
| M356T262 | | | up | | | | | |
| M374T262 | | | up | | | | | |
| M377T262 | | | up | | | | | |
| M378T262 | | | up | | | | | |
| M161T263 | | down | down | | down | | | |
| M177T263 | | down | up | | | | | |
| M179T263 | | down | | | down | | | |

| | | | | | | | | |
|-----------|------|------|------|------|------|------|------|------|
| M195T263 | | down | | | down | | | |
| M205T263 | | down | down | | down | | | |
| M223T263 | | down | | | | | | |
| M241T263 | | down | | | down | | | |
| M263T263 | | down | | | down | | | |
| M439T264 | | | | | | | | up |
| M171T275 | | | | | | down | | |
| M163T287 | | | up | up | | | | |
| M377T287 | | | up | | | | | |
| M211T293 | | | up | | | | | up |
| M295T304 | | | up | | | | | |
| M469T304 | | | up | | | | | |
| M315T312 | up | up | | up | up | up | up | up |
| M480T317 | | | up | | | | | |
| M110T320 | up | up | up | up | | | up | |
| M224T320 | up | up | up | up | up | | up | up |
| M210T321 | up | up | up | up | | | up | |
| M303T352 | | | down | down | | | | |
| M280T354 | up | up | up | up | up | | up | up |
| M281T354 | up | up | up | up | up | | up | up |
| M366T354 | | | | | | | | up |
| M184T355 | | | | | | | | up |
| M219T355 | | | | up | | | | |
| M525T376 | | | | | | | down | |
| M177T379 | down | | down | down | down | down | | |
| M312T379 | down | | down | down | | | | |
| M352T379 | | | down | down | | down | | |
| M548T382 | | | up | | | up | | up |
| M619T382 | | | | | up | | | |
| M359T384 | | | | | | | down | |
| M1035T385 | | | | | | | down | |
| M369T394 | | | | | | | | up |
| M594T394 | | | | | | | down | |
| M595T394 | | | | | | | down | |
| M1051T394 | | | | | | | down | |
| M185T395 | | | | | | | | up |
| M368T395 | | | | | | | | up |
| M537T395 | | | | | | down | down | |
| M305T396 | | | down | down | | down | down | |
| M145T409 | | down | | | | | | down |
| M295T409 | | | | | | | down | |
| M296T409 | | | | | | | down | |

| | | | | | | | | |
|-----------|------|------|------|------|------|------|------|------|
| M325T409 | | | | | | | down | |
| M326T409 | | | | | | down | | |
| M432T411 | | | | | | down | down | |
| M537T411 | | | | down | | down | down | |
| M594T411 | | | | | | down | down | |
| M1051T411 | | | | | | down | down | |
| M209T418 | up | up | up | | | up | up | up |
| M218T420 | | | up | | | | | |
| M325T425 | down | | | | | | | |
| M588T432 | | | | | | down | down | |
| M572T437 | | | | | | down | down | down |
| M527T446 | | | | | | down | down | down |
| M574T446 | | | | | | down | down | down |
| M289T448 | | | | down | | | | |
| M543T452 | | | down | down | | down | down | down |
| M688T453 | up | | up | up | up | down | up | up |
| M557T455 | | | | | up | | | |
| M634T455 | | | | | up | | | |
| M580T461 | | | down | down | | down | down | |
| M97T462 | down | | | | down | | | |
| M115T462 | down | | down | | | | | |
| M223T462 | down | | down | | down | | | down |
| M241T462 | | down | | | down | | | |
| M259T462 | | down | | | down | | | |
| M260T462 | down | down | | | | | | |
| M277T462 | down | | | | down | | | |
| M289T462 | | | | down | | down | down | down |
| M295T462 | down | | | down | | down | down | down |
| M296T462 | down | down | | | | down | down | down |
| M355T462 | | | | down | | | | |
| M358T462 | down | down | | | down | | | down |
| M364T462 | | | | | | down | | |
| M370T462 | | | | down | | down | | down |
| M414T462 | | | | down | | down | | down |
| M415T462 | | | down | down | | down | | down |
| M416T462 | down | | down | down | | down | down | down |
| M421T462 | | down | down | down | | down | down | down |
| M436T462 | | | | down | | down | | down |
| M457T462 | | | | | | down | | |
| M458T462 | | | | | | down | | |
| M525T462 | | | | down | | | | |
| M528T462 | | | | down | | down | | |

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|-----------|------|------|------|------|------|------|------|------|
| M529T462 | | | | | | down | | down |
| M539T462 | | | | | down | | | |
| M559T462 | | | | | | down | | down |
| M576T462 | | | | down | | down | | down |
| M577T462 | | | | | | down | | |
| M1033T462 | | | | down | | down | | down |
| M1034T462 | | | | down | | | | |
| M284T466 | up |
| M306T466 | up |
| M371T469 | | | | | | | down | |
| M368T470 | down | down | | | | | | |
| M636T470 | | | | | up | | | |
| M1065T470 | | | down | | | down | | down |
| M319T471 | | | | | up | | | |
| M400T471 | | | | | up | | | |
| M555T471 | | | | | up | | | |
| M558T471 | | | | | up | | | |
| M569T471 | | | | | | up | | up |
| M589T471 | | | | | up | | | |
| M637T471 | | | | | up | | | |
| M638T471 | | | | | up | | | |
| M739T471 | | down | | | | down | | |
| M1093T471 | | | | | up | | | |
| M85T479 | | | | | down | | | |
| M277T479 | | | | | up | | | |
| M903T479 | | | | down | | down | down | |
| M91T480 | down | | | | down | | | |
| M97T480 | down | | | | down | | | |
| M109T480 | down | | | | down | | | |
| M115T480 | down | | | | down | | | |
| M116T480 | down | | | | down | | | |
| M127T480 | down | | | | down | | | |
| M128T480 | down | | | | down | | | |
| M133T480 | down | down | | | down | | | |
| M134T480 | down | | | | | | | |
| M146T480 | down | down | | | down | | | |
| M164T480 | down | | | | | | | |
| M193T480 | down | | | | down | | | |
| M199T480 | down | | | | down | | | |
| M204T480 | | | | | down | | | |
| M217T480 | down | | | | down | | | |
| M223T480 | down | | | | down | | | |

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|----------|------|------|------|------|------|------|------|------|
| M224T480 | | | | | down | | | |
| M229T480 | down | | | | down | | | |
| M241T480 | down | | | | down | | | |
| M242T480 | down | down | | | down | | | |
| M259T480 | down | | | | | | | |
| M260T480 | down | down | | | down | | | |
| M261T480 | down | down | | | down | | down | |
| M278T480 | down | down | | | | down | | |
| M291T480 | | | | | | | down | |
| M295T480 | down | | down | | | | down | |
| M298T480 | down | down | | | | down | down | |
| M328T480 | | | | | | | down | |
| M356T480 | | | | | | | down | |
| M357T480 | | | | down | | | down | down |
| M358T480 | down | | | | | | | |
| M364T480 | | | | | down | | | |
| M439T480 | | | | | | | down | down |
| M459T480 | | | | | | | down | |
| M460T480 | | | | | | | down | |
| M510T480 | down | down | down | down | | down | down | down |
| M527T480 | | | down | down | | | down | |
| M529T480 | | | | | | | | down |
| M537T480 | down | | | | | | | |
| M582T480 | | | | | | | down | |
| M742T480 | down | down | down | | | down | down | down |
| M563T481 | | | | down | | down | | down |
| M571T481 | | | down | | down | | | |
| M314T485 | | up | | | | | | |
| M336T485 | up | up | | up | up | | | |
| M414T490 | down | | | down | | | down | down |
| M149T493 | | up | | up | | | | |
| M259T499 | down | down | down | | down | down | down | down |
| M417T499 | down | down | down | down | | down | down | down |
| M471T505 | | | | | | | | up |
| M578T514 | | | | | | down | down | |
| M579T514 | | | | | | down | down | |
| M416T517 | | | | down | | | | |
| M325T557 | | | | down | | | down | |
| M145T584 | | | | | down | | | |
| M213T589 | | | | | | | up | |
| M140T613 | | | up | | | | up | |
| M149T613 | | | | up | | | | |

| | | | | | | | | |
|-----------|----|----|------|------|------|------|------|------|
| M163T613 | | | | | | | up | |
| M177T613 | | | | | down | | down | |
| M218T613 | | | | | | | down | |
| M308T613 | | | | | | | | up |
| M321T613 | | | | up | | | | |
| M416T624 | | | | down | | | down | |
| M410T636 | | | | | | up | | up |
| M293T645 | | | down | down | | | | |
| M295T664 | | | | down | | down | | down |
| M625T664 | | | down | down | | down | down | down |
| M1254T664 | | | down | | | | | |
| M474T668 | up | up | | | up | | | up |
| M277T674 | | | down | down | | | | |
| M353T674 | | | down | down | | down | | down |
| M158T677 | | | up | | | | | up |
| M211T679 | | | | | down | | | |
| M414T681 | | | | | | | | down |
| M290T685 | | | | | | | | down |
| M333T704 | | | down | | | | down | down |
| M245T706 | | | up | | | | | |
| M318T714 | up | up | up | up | up | up | up | up |
| M311T716 | | | | | | down | | |
| M325T717 | | | | down | | down | | |
| M295T720 | | | down | | | | | down |
| M331T720 | | | | | | | up | |
| M335T720 | | | down | | | | down | down |
| M381T720 | | | | | | | down | down |
| M698T720 | | up | | | | down | | |
| M716T720 | | | | | | down | | |
| M738T720 | | | | | | down | | |
| M783T720 | | | | | | | | up |

2.2.2. Tomato root metabolomics analyses between nematode-inoculated (1 pest) plants and multiple-pest-infested plants

The root metabolomics between one nematode-infested plants and multiple pest-infested plants were not significantly different. Totally, only 29 melocules were found between the plant solely infested by nematodes and the plant infested by nematodes and other aboveground pests. Metabolic compounds in tomato roots changed greatest (16 significant features) when tomatoes were attacked by nematodes and aphids.

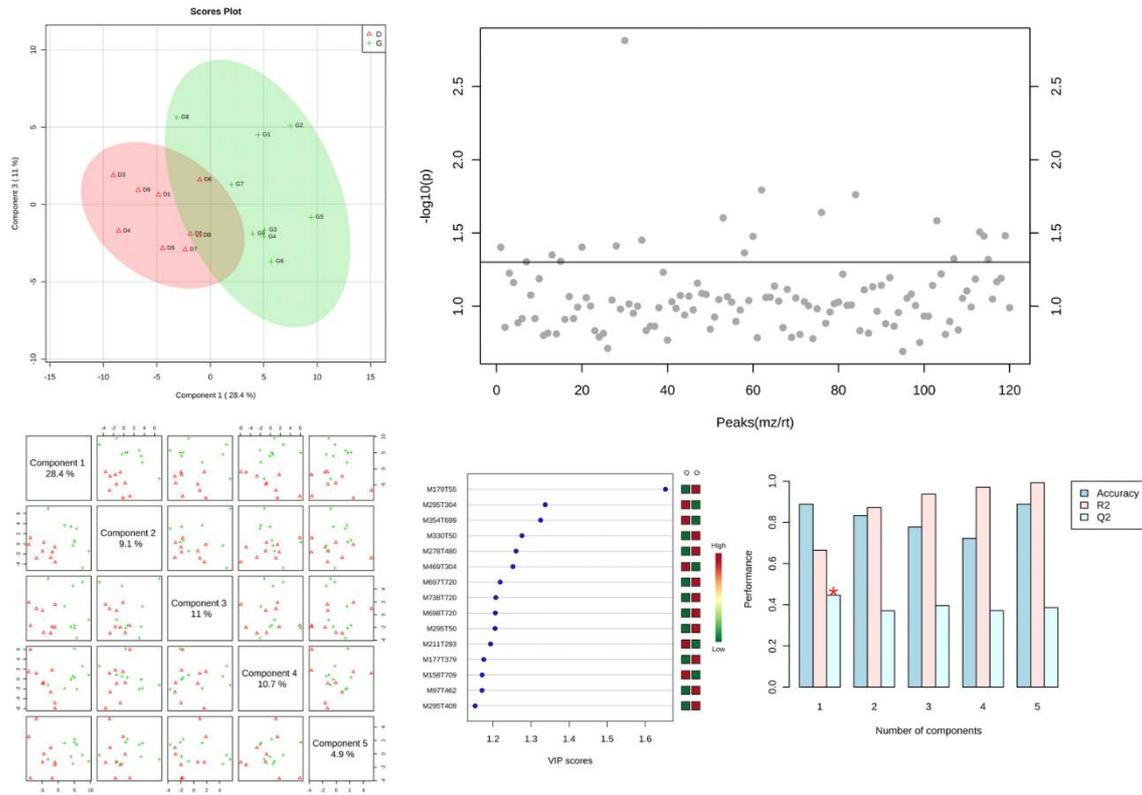


Figure 4. Roots metabolomics analyses between the plants (in Treatment D) inoculated by nematodes and plants (in Treatment G) infested by nematodes and cotton bollworms.

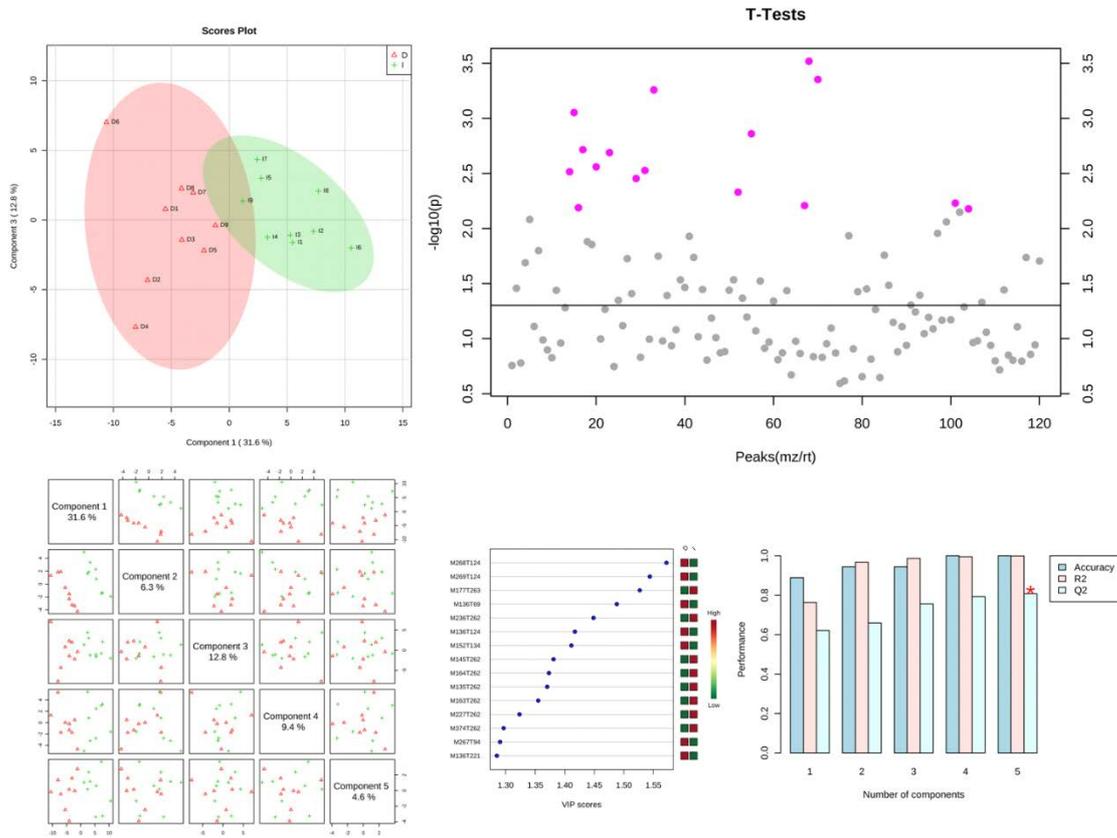


Figure 5. Roots metabolomics analyses between the plants (in Treatment D) inoculated by nematodes and plants (in Treatment I) infested by nematodes and aphids.

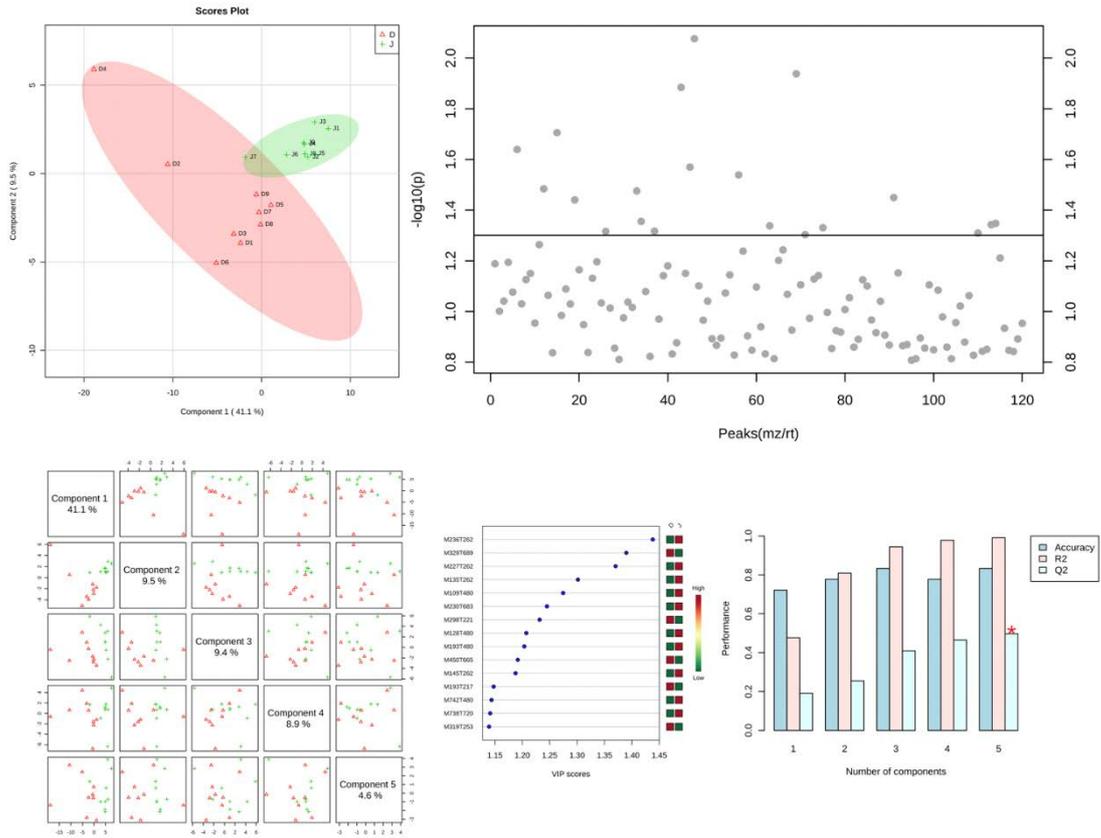


Figure 6. Roots metabolomics analyses between the plants (in Treatment D) inoculated by nematodes and plants (in Treatment J) infested by nematodes and powdery mildew fungi.

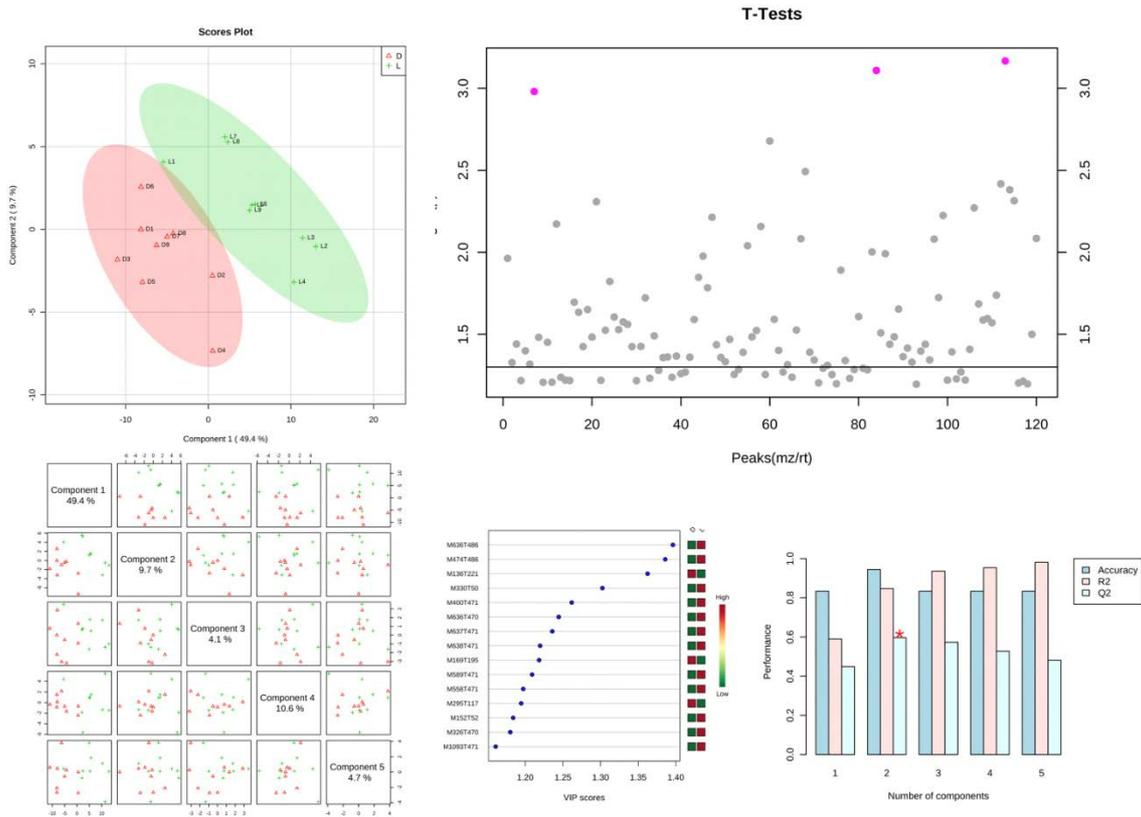


Figure 7. Roots metabolomics analyses between the plants (in Treatment D) inoculated by nematodes and plants (in Treatment L) infested by nematodes and cotton bollworms and aphids.

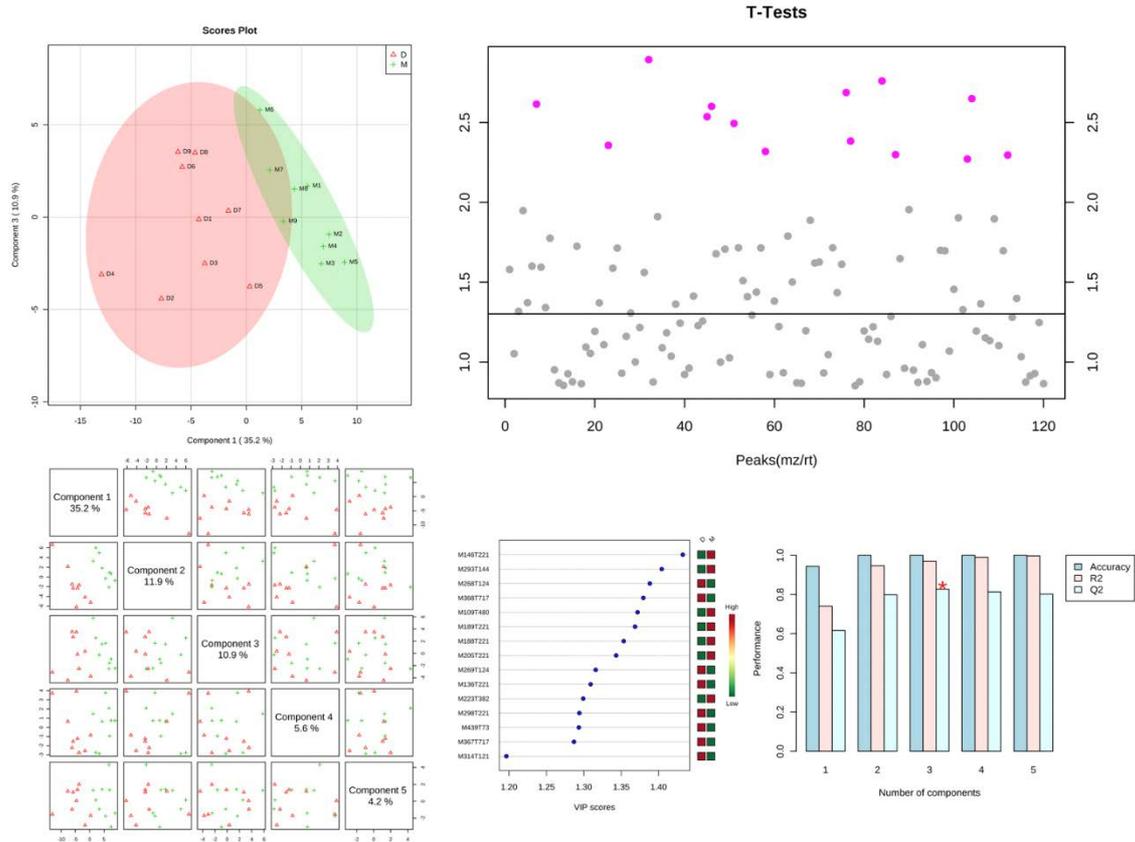


Figure 8. Roots metabolomics analyses between the plants (in Treatment D) inoculated by nematodes and plants (in Treatment M) infested by nematodes, cotton bollworms and powdery mildew fungi.

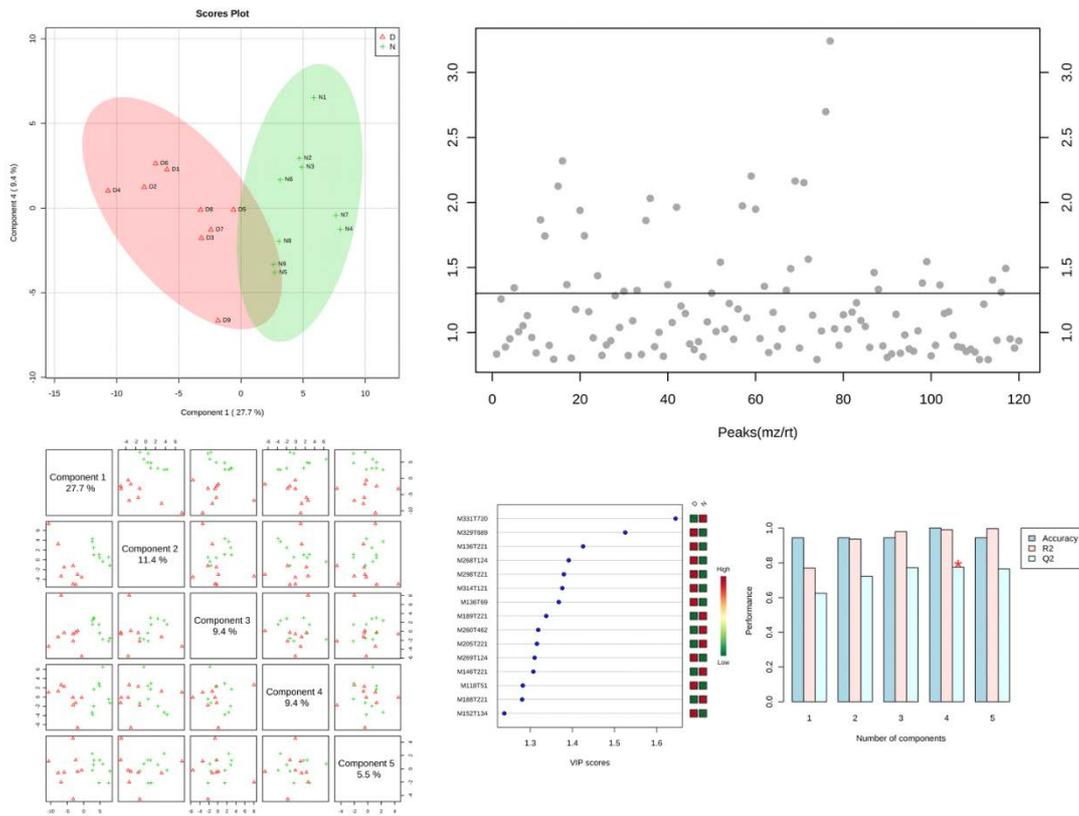


Figure 9. Roots metabolomics analyses between the plants (in Treatment D) inoculated by nematodes and plants (in Treatment N) infested by nematodes, aphids and powdery mildew fungi.

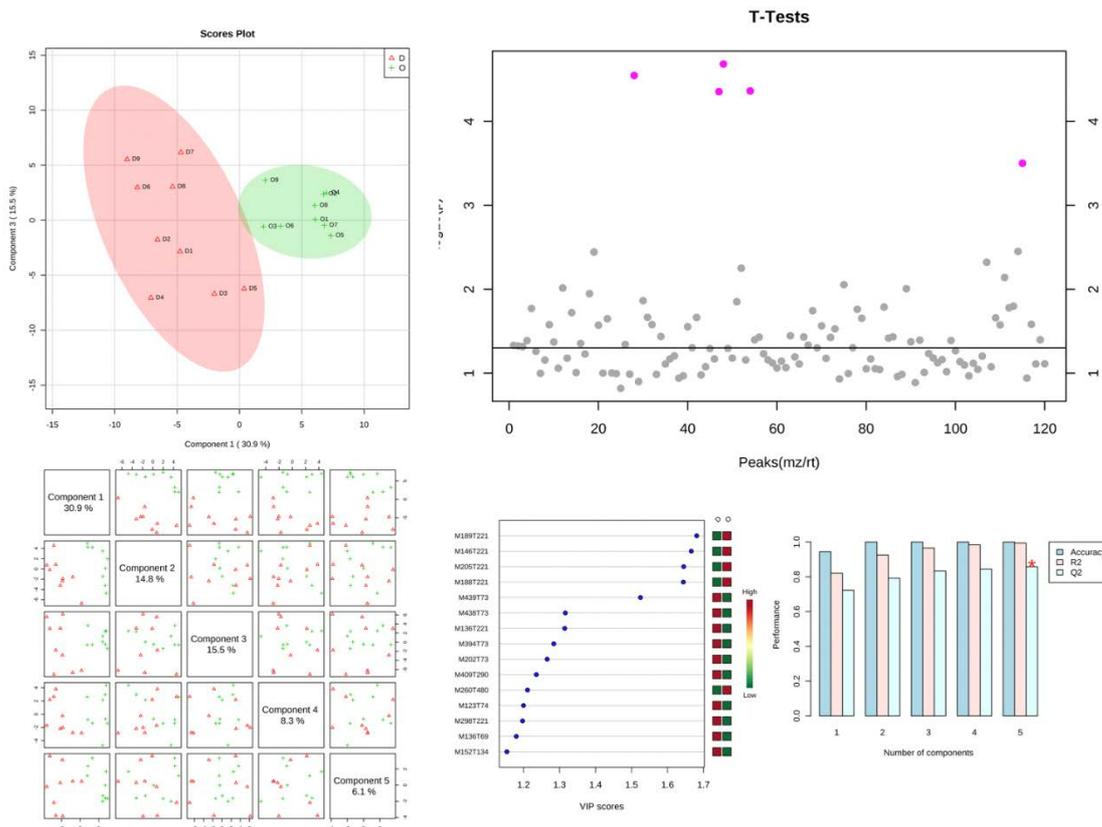


Figure 10. Roots metabolomics analyses between the plants (in Treatment D) inoculated by nematodes and plants (in Treatment O) infested by nematodes, cotton bollworms, aphids and powdery mildew fungi.

Table 2. Significant features detected on roots between nematode-inoculated plants and multiple-pest-infested plants. Me: *M. euphorbiae*; On: *O. neolycopersici*; Mi: *M. incognita*; Ha: *H. armigera*

| 1 pest (nematode) | 2 pests | | | 3 pests | | | 4 pests |
|------------------------------------|------------|------------|------------|---------------|---------------|---------------|------------------|
| treatment | G Mi+Ha | I Mi+Me | J Mi+On | L Mi+Ha+Me | M Mi+Ha+On | N Mi+Me+On | O Mi+Ha+Me+On |
| The number of significant features | 0 | 16 | 0 | 3 | 14 | 0 | 5 |
| M136T69 | | down | | | | | |
| M439T73 | | | | | down | | down |
| M267T94 | | down | | | | | |
| M136T124 | | down | | | | | |
| M268T124 | | down | | | down | | |
| M269T124 | | down | | | down | | |

| | | | | | | | |
|----------|--|------|--|------|------|--|----|
| M152T134 | | down | | | | | |
| M293T144 | | | | | up | | |
| M136T221 | | down | | down | down | | |
| M146T221 | | | | | up | | up |
| M188T221 | | | | | up | | up |
| M189T221 | | | | | up | | up |
| M205T221 | | | | | up | | up |
| M298T221 | | | | | down | | |
| M135T262 | | up | | | | | |
| M145T262 | | up | | | | | |
| M163T262 | | up | | | | | |
| M164T262 | | up | | | | | |
| M227T262 | | up | | | | | |
| M236T262 | | up | | | | | |
| M374T262 | | up | | | | | |
| M378T262 | | up | | | | | |
| M177T263 | | up | | | | | |
| M223T382 | | | | | up | | |
| M109T480 | | | | | up | | |
| M474T486 | | | | up | | | |
| M636T486 | | | | up | | | |
| M367T717 | | | | | down | | |
| M368T717 | | | | | down | | |

3. Volatile metabolomics analyses of tomato plants under above-below ground indirect interactions in a tri-trophic system

3.1. Material and methods

3.1.1. Experimental design

The same organism infestation and schedule in Chapter 4 was followed by this experiment. The collections of VOCs were conducted in the two groups with parasitoids release. Constitutive volatiles (the 1st collection in control) and induced volatiles (the 1st collection in treatment) by nematodes and powdery mildew fungi was respectively trapped on the day before aphid release. Twelve days after the aphid release, the 2nd volatile collection was conducted as the short-term induction in control and treatment, respectively. The 3rd collection for the long-term volatile induction was performed on the 27th day after aphid release and before parasitoid release. The volatile trapping in each control and treatment group was replicated 5 times.

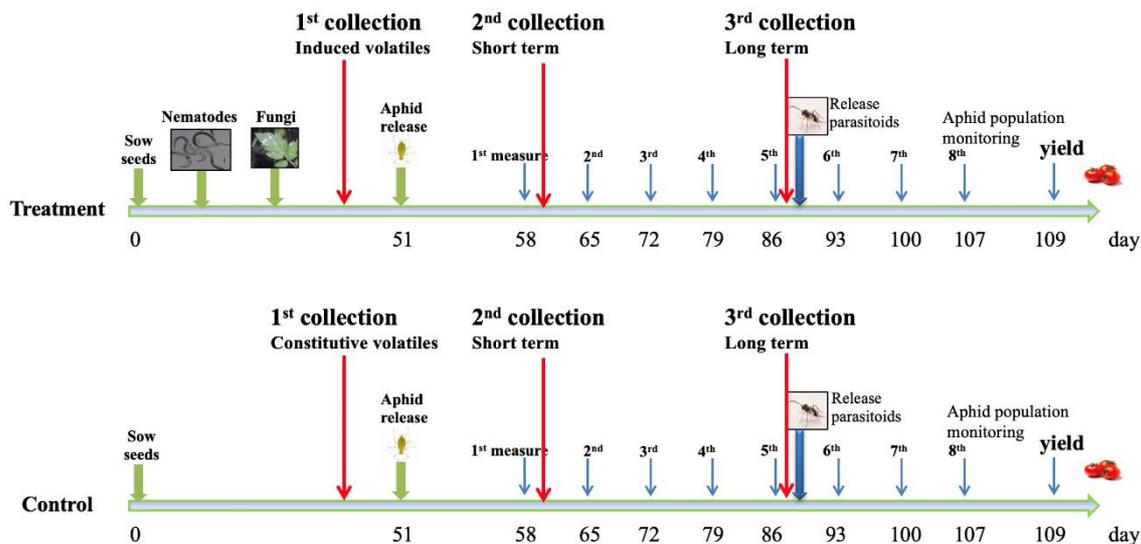


Figure. 11 Experimental design for VOCs collection.

3.1.2. Plant VOCs collection

Solid phase micro-extraction (SPME) fibers were chosen to extract plant VOCs. A high sensitivity can be detected with this technique. Volatile organic compounds emitted locally by tomato leaves were collected from the aphid-infestation leaves (Control) or three-pest-infestation leaves via SPME (Supelco, Bellefonte, USA,) using gray fibers (50/30 μm , DVB/CAR/PDMS, 24 Ga). This adsorbent phase is a mixture of divinylbenzene, carboxen, and polydimethylsiloxane, covering a broad range of polarities (Supelco producer information). Odorless Nalophan bags, all the same size (15 x 30 cm), were put around the fifth leaves (5 leaflets) from the top of tomato plants to be sampled and closed tightly using teflon tape creating headspaces of comparable sizes. The SPME-fiber was inserted by a small cut in the side of the bag, and exposed for 30 minutes. Five replicates were collected from both treatment and control.

3.1.3. Plant VOCs analyzed by GC-MS

SPME-fibers were analyzed by GC-MS (Hewlett Packard, HP 6890 Series: Gas Chromatograph and Mass Selective Detector). Desorption temperature was 250 $^{\circ}\text{C}$ for 5 min and the injection was splitless with helium gas. The temperature gradient of the column oven started at 40 $^{\circ}\text{C}$, was held for 5 minutes, and afterwards heated to 70 $^{\circ}\text{C}$ with 1.5 $^{\circ}\text{C}/\text{min}$, to 150 $^{\circ}\text{C}$ at 3 $^{\circ}\text{C}/\text{min}$, and to 250 $^{\circ}\text{C}$ at 25 $^{\circ}\text{C}/\text{min}$, and held for 2.33 min with a flow of 0.7 ml/min. Chromatograms were evaluated using Agilent Enhanced ChemStation E.02.02.1431.

3.1.4. Data analysis and Results

All volatile samples were analysed by the GC-MS and chromatograms were obtained as shown below.

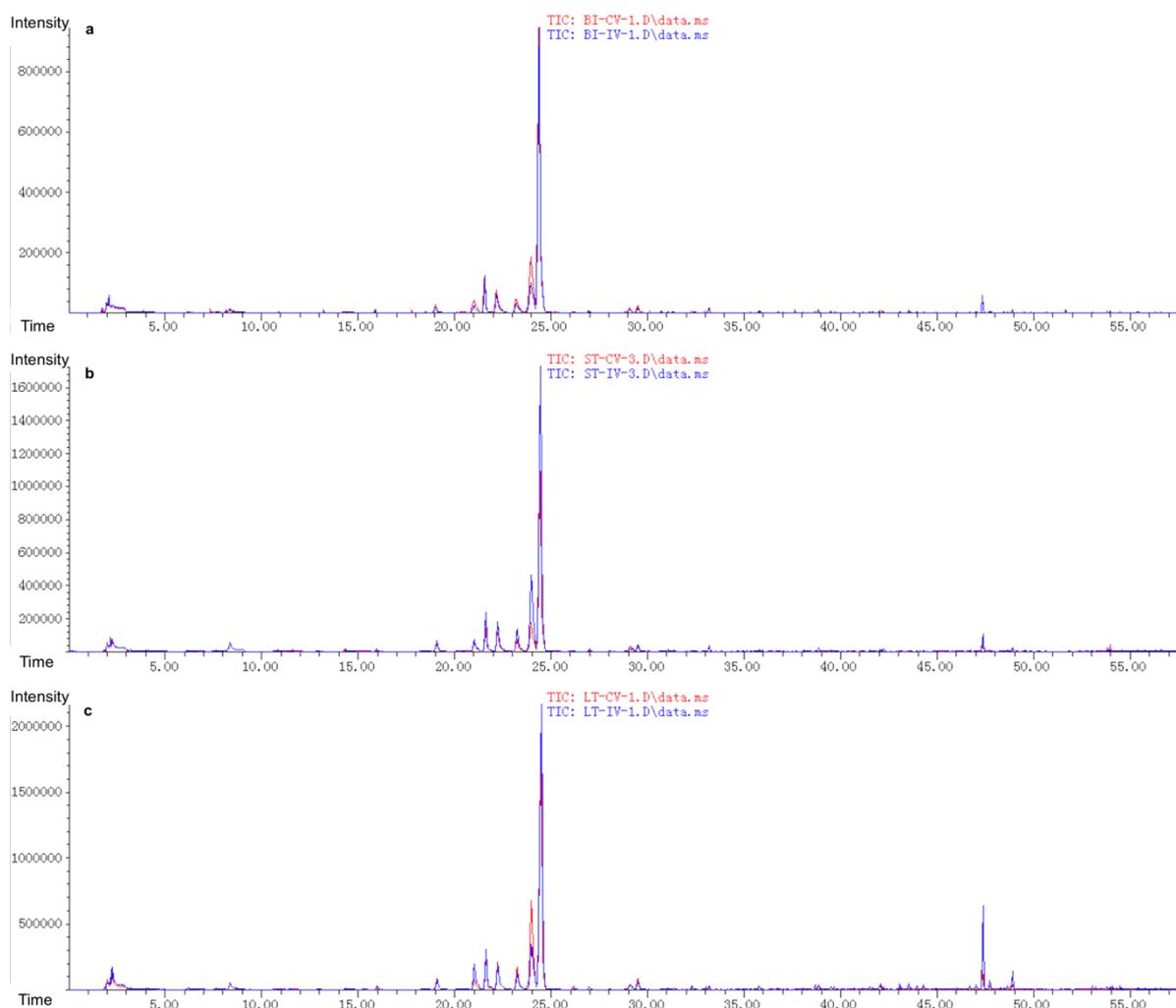


Figure. 12 Chromatograms in the Control (red line) and Treatment (blue line) at different time. a: volatiles trapped before aphid infestations (1st collection in Fig. 11); b: volatiles trapped at short term after aphid infestation (2nd collection in Fig. 11); c: volatiles trapped at long term after aphid infestation (3rd collection in Fig. 11).

Globally, it appears that blends of volatiles emitted by the plant infested solely by aphids (red lines) and the plant infested by multiple pests (blue lines) were different. However, further statistical analysis of comparison between constitutive volatiles and induced volatiles are still in progress.

4. Discussion

The root metabolomics of tomato plants could be remarkably altered by the pest infestation from the results of metabolomics analyses between control and treatment plants. Changes in

quantity of significant molecules of the treatment plants infested by the different number of pest species was similar, compared to control. Totally, 316 significant molecules were observed in the roots between control plants and pest-infestation plants. Both up-regulated and down-regulated molecules were detected in the pest-infestation roots (Table 1). Moreover, the altered molecules whether up-regulated or down-regulated in different treatments showed almost same variation trends. Only 4 molecules were changed differently in different pest-infestation plants, compared to control. In addition to the 4 molecules, there are 146 molecules up-regulated and 166 molecules down-regulated in pest-infestation plants.

Especially the molecules at the retention time of 73seconds (T73), 78s (T78), 110s (T110), and 239s (T239) were almost up-regulated in all the pest-infestation roots (Fig. 3c; Table 1). In addition, molecules at the retention time of 108s (T108) and 166s (T166) are both down-regulated in all the treatment roots compared to the root without nematode infestation. The compounds separated by UHPLC earlier than 159s (retention time <159s) were mainly up-regulated in tomato roots after nematodes infection. However, compounds that were separated by UHPLC later than 379s (retention time > 379s) are mainly down-regulated in tomato roots after nematodes infestation.

When the root metabolomics were analyzed among the nematode-attack plants and the plants jointly attacked by multiple pests, i.e. 2 pests (Fig. 4; Fig.5; Fig. 6), 3 pests (Fig. 7; Fig. 8; Fig. 9) and 4 pests (Fig. 10), no integral difference in the root metabolites was found. In addition, only 29 significant molecules were detected between the roots solely inoculated by nematodes and the roots infested by multiple pests. It indicated the alteration in root metabolism of pest-infestation plants depend majorly on the nematode inoculation. Moreover, the distinct molecules observed between nematode-inoculated plants and multiple pest-infestation plants were as well involved in the metabolic modulations of plants induced by these pests. This indicated the participant of above-ground pests just caused quantitative changes in a few metabolic molecules. The significant molecules, especially the distinct ones among different pest-infestation plants should be further identified and validated for their biological activities.

CHAPTER 6. GENERAL DISCUSSION

Based on ‘plant-mediated indirect interactions’, my Ph.D. study mainly focused on the modulating factors, impact on the community, and underlying phytochemical mechanisms in the tri-trophic system. Both biotic (pest biodiversity, feeding guilds, attacking sequence, attacking location and host specialization) and abiotic (sublethal effects of pesticides) factors which modulated such indirect interactions were considered in this work. Investigations were conducted at both individual (life-history parameters) and population levels, involving the first trophic level (plants), the second trophic level (pests) and the third trophic level (parasitoids). Plant metabolomics were analyzed to tentatively explain the potential mechanisms resulting in such ‘plant-mediated indirect interactions’ at phytochemical levels. The main findings in my study were discussed as follows.

1. At individual level

Five biotic factors, including the number of pest species (1-4 pest species), feeding guilds (chewing caterpillars, sap-feeding aphids, powdery mildew fungi and root knot nematodes), attacking sequence (simultaneous attack/sequential attack), attacking location (shoot/root) and host specialization (specialist/generalist), and **one abiotic factor** (sublethal effects of beta-cypermethrin) were involved to evaluate the influence **on the life-history traits of each pests and plant fitness**.

>A general relationship was obtained between the pest species number involved in plant-mediated indirect interactions and insect performances as well as plant fitness.

When these different pest species attacked tomato plants **at the same time**, insect performances were **positively correlated with** the pest species number in indirect interactions (Fig. 4 in Article 1), i.e. the more pest species were involved in the plant-mediated indirect interactions, the more number of aphid individuals and the faster development of caterpillar larva were on tomatoes. When the species attacked tomato plants **in sequence**, insect performances **were negatively correlated with** the abundance of indirect interactions (Fig. 4 in Article 2), i.e. the more pest species involved in such indirect interactions, the less number of aphid individuals and the slower development of caterpillar larvae on the plant.

The distinct relationship between pest species number and herbivore performance may be due to the trade-offs employed by plants between on plant defense and plant growth, and on

constitutive defense vs induced defense, respectively (Cipollini et al., 2018). From the perspective of evolution, it would appear to allocate more of their resources to growth, to develop or to reproduce than to defenses, especially for constitutive defenses, because they are considered as costly for plants (Coley, 1987; Herms and Mattson, 1992). Alternatively, induced defenses should be quickly (induced defenses could be triggered in several hours) triggered in order to minimize the allocation cost, compared to constitutive defenses, after plants were attacked by pests (Coley, 1987; Guo and Ge, 2017; Karban and Baldwin, 1997). Also, the induced defenses could produce and/or accumulate a variety of secondary metabolites, which are considered more effective due to the direct effects on pest growth and development (Karbon, 2011; Kariyat et al., 2013; Kaur et al. 2015). Therefore, insects benefited from the indirect interactions involving more different pest species when they simultaneously attacked the host, which may be due to the less resistance of the host to defend against multiple pests at the same time. Namely, plants are easier to be attacked by more pest species' simultaneously attack because it would be difficult to the host to defend against more different pest species than to that to defend against fewer species at the same time. The more pest species simultaneously attacked the host, the less magnitude of plant defenses would be assigned to each species.

On the opposite, when multiple pests successively attacked tomato plants, defensive compounds produced by induced defenses may gradually accumulate with the increase of attack by pest species (Mewis et al., 2006). Hence, when pest successively infested hosts, the pest exposed to more abundant indirect interactions should suffer more by the multiple induced defenses of plants, compared to the pest that was subjected to a simple interaction involving fewer pest species (Eyles et al., 2010; Karban, 2011). Especially, the performance of herbivorous insects, i.e. aphid and caterpillars, which arrived later than the other two pathogenic organisms, i.e. mildew fungi and nematodes, were more able to be negatively impacted.

When tomato plants are sequentially attacked by multiple pests, the plant growth including plant height and plant nodes was also positively correlated with the number of pest species involved in such indirect interactions (Fig. 6 in Article 2). Such a growth acceleration in the plants caused by the more complex interactions among multiple pest species could be a compensation of plant fitness as a response to multiple pest attack (Boege, 2005; McNaughton, 1983).

However, we did not find a correlation between performances of pathogenic organisms and the number of pest species involved in indirect interactions, no matter when the hosts were attacked by pests simultaneously or sequentially. This could be linked to the different

mechanisms in plant defenses against herbivorous insects and pathogenic organisms (Jones and Dangl, 2006; War et al., 2018).

>*The feeding strategy of pests has influences on the outcome of plant-mediated indirect interactions among pests.*

Four different feeding guilds of pests, i.e. chewing caterpillars (*S. exigua/H. armigera*), sap-feeding aphids (*M. euphorbiae*), powdery mildew fungi (*O. neolycopersici*), and root-knot nematodes (*M. incognita*), were involved in my Ph.D. study to investigate the plant-mediated indirect interactions among them. Historically, JA (jasmonic acid) was supposed to be involved in defense against chewing-herbivores, while SA (salicylic acid) was considered to be linked to the defense against biotrophic pathogens and piercing-sucking insects (Biere and Goverse, 2016). Both JA and SA could be involved in the induced defenses against root-knot nematodes, depending on different plant species (Guo and Ge, 2017; Nahar et al., 2011). In tomato, the significant accumulation of SA, instead of JA, was observed in roots after *M. incognita* inoculation (Guo and Ge, 2017). The two main hormonal pathways were commonly antagonistic with each other, and therefore the outcome of plant-mediated indirect interactions could be predicted to some extent (Biere and Goverse, 2016). Our data support these predictions but only partly. When two species simultaneously attacked tomato plants, the number of aphid individuals was negatively affected by the co-infestation with caterpillars and fungi, but was not impacted by root-knot nematodes (Fig. 1c in Article 1). However, when two species sequentially attacked tomato plants, aphid performance was not affected by all the other three pests (Fig. 1c in Article 2).

In fact, it would be some opportunistic if the outcome of plant-mediated indirect interaction was predicted only depending on feeding guilds of plant attackers. Firstly, the interaction between phytohormonal pathways is not the only mechanism which decides plant-mediated indirect interactions (Biere and Goverse, 2016). Such indirect interactions also rely on resource reallocation within plants and alteration of sink-resource relationship (Biere and Goverse, 2016). Secondly, in addition to the feeding strategy of pests, the induction of phytohormonal signaling may be also linked to the specific pests, the diet specialization and host species (Figon, 2013; Leon-Reyes et al., 2010a, b). For example, *S. exigua* as a chewing caterpillar, was reported to activate the SA signaling pathway in *N. attenuate* (Figon, 2013). In addition, SA is typically prioritized over JA in *Arabidopsis*, but plants could use ET to fine tune defenses by prioritizing JA induction over SA in response to multiple pests (Leon-Reyes

et al., 2010a, b). Thirdly, two different phytohormonal signalings could be induced by one attacker on the same host. Both the SA and JA pathways are induced after plants were infected by *Fusarium* spp., a hemibiotrophic fungal pathogen (Ding et al., 2011). Whereas, SA was triggered early due to its importance to establish resistance, JA followed to promote resistance during later time points (Ding et al., 2011). Thus, the influence and importance of pest feeding guilds to plant-mediated indirect interactions should be considered in a certain plant-attacker circumstance.

>The interaction between belowground and aboveground pests could be either unidirectional or bidirectional, which as well depend on attacking sequence and pest biodiversity.

When **belowground nematode arrived earlier** than aboveground pests, **nematodes did not be affected** by the other aboveground pests which attacked tomato later (Article 2). However, when nematodes as well as aboveground pests **attacked tomato plants at the same time**, the above-belowground interactions depend on **the species number** presence on the same host (Article 1). When 2 pests simultaneously attacked tomatoes, aboveground pests unidirectionally affected belowground nematodes, i.e. the aboveground pests impacted both aboveground and belowground pests, but they were not affected by belowground nematodes (Fig. 1 in Article 1). However, when 3 pests simultaneously attacked tomatoes, **both** belowground nematodes and aboveground pests **were affected by each other** (Fig. 2 in Article 1). Our results support the **Spatio-temporal pattern** of plant-mediated indirect interactions, i.e. such indirections are temporal and spatial-dependent.

The order of pest arrival can be an important determinant of plant-mediated indirect interactions between aboveground and belowground pests (Erb et al., 2011). The early attacker on the host may be as one of important strategies of pests to avoid competition with other plant challengers. Erb et al. also reported that the outcome of interactions between the aboveground herbivorous larvae of *Spodoptera frugiperda* and belowground phytophagous larvae of *Diabrotica virgifera* varied with their attacking sequence on the teosinte (Erb et al., 2011). In addition, according to the organ-specific immune responses, the organ (shoot or root) where the above-belowground interaction is initiated play an important role in shaping the outcome of the interactions (Balmer and Mauch-Mani, 2013). Different location of inducer and responder would result in the asymmetry in above-belowground interactions (Biere and Goverse, 2016).

Moreover, the asymmetry in plant-mediated indirect interaction caused by the different attacking sequence of pests as well occurs in the same plant organ. For example, when powdery mildew fungi and aphids attacked tomato plants at the same time, fungal reproduction benefits from such interactions, but the number of aphid individuals on the same host significantly decreased (Fig. 1b, 1c in Article 1). Nevertheless, when fungi infested tomatoes before aphids, the performance of both the two species of pests were not impacted (Fig. 1 in Article 2). Similar results were reported by Soler et al (2012b). When aphids *B. brassicae* and caterpillars *P. brassicae* attacked *B. oleracea* together, caterpillars benefited more from their simultaneous attack than the sequential attack by the two species of herbivores.

>*The abiotic factor (sublethal effects of beta-cypermethrin) modulates the interspecific interaction between the specialist A. glycines and generalist A. solani on soybean plants.*

The specialist aphid *A. glycines* had better performance (higher fecundity) than the generalist aphid *A. solani* on soybean, likely due to the better adaption of the specialist pest to the host defense (Agrawal and Kurashige, 2003; Travers-Martin and Müller, 2007). Following a co-evolution process, *A. glycines* as the specialist aphids on soybeans has evolved more physiological adaptations to cope with the plant constitutive defenses, e.g. through the detoxification or the sequestration of plant toxins (Ali and Agrawal, 2012; Barrett and Heil, 2012; Florent, 2013). When the two species of aphids attacked soybeans sequentially, fecundity of the later arriving aphids was suppressed by the early arrivals. This is likely linked to the accumulation of isoflavonoids that act as toxins against the subsequent aphids on the soybean (Hohenstein et al., 2019). However, such interspecific interactions are asymmetric between the specialist aphids and generalist aphids. The specialist aphid performance suffered more from the interspecific competition. In addition, pre-infestation by one aphid species on the soybean facilitate feeding of the other aphid species on the host (Fig. 3 in Article 3). Such induced susceptibility of hosts to the interspecific aphid could benefit from some effectors secreted from aphid salivary to overcome the host defenses (Hohenstein et al., 2019; Yates and Michel, 2018).

Another similar asymmetric interaction was observed between the generalist *Trichoplusia ni* and the specialist *P. xylostella* on the wild radish (Agrawal, 2000). In this study, the specialist caterpillar caused a resistance on the host to the generalist caterpillar, but the specialist caterpillar was not impacted by the generalist (Agrawal, 2000). Such different asymmetric

interaction between specialist and generalist pests on soybean and on the wild radish may result from the distinction in the feeding modes between aphids and caterpillars.

Beta-cypermethrin is one of the widely-used pesticides for aphid control in the field. It has been reported to cause an array of sublethal effects on the targeted pest, including stimulation on fecundity (Qu et al., 2017). This could be a potential cause of pest resurgence after its application in the field (Wang et al., 2008). In our results, sublethal concentration of beta-cypermethrin accelerate the interspecific competition between the specialist aphids and the generalist aphids, by multiplying the inhibition of aphid abundance. Exposure to the sublethal concentration of beta-cypermethrin, the two species of aphids need to spend more energy to detoxify pesticides and migrate within the host (Fig. 2 in Article 3), which may lead to the reduction of aphid fecundity. Furthermore, the induced susceptibility was as well impaired by exposure to sublethal beta-cypermethrin, which could result from the repellent and antifeedant activities of pyrethroid (Bandason, 2018; Diabate et al., 2014).

2. At population level

The aphid population dynamic involved in the above-belowground indirect interactions and its **biological control efficiency** by parasitoids were monitored **in a tri-trophic system**.

>Above-belowground indirect interactions among nematodes, fungi and aphids slow down the aphid long-term population.

Interestingly, we found that the number of aphid individuals was always suppressed, whether when the three pests (nematodes, fungi and aphids) attacked tomato plants simultaneously or sequentially (Fig. 2c in Article 1; Fig. 2c in Article 2) at one generation scale. Similar result was as well achieved on the soybean system, when the cyst nematode *H. glycines*, the fungus *C. gregata* and the soybean aphid *A. glycines* attacked both soybean cyst nematode-susceptible and soybean cyst nematode-resistant cultivars (McCarville et al., 2012). The aphid population was significantly suppressed on the soybean infested by multiple pests compared to that on the soybean solely infested by aphids, disregarding the susceptibility of the cultivar (McCarville et al., 2012). A strong and stable interactions should exist between nematodes, fungi and aphids, which contributes to a negative effect on aphid performance.

Moreover, the long-term influence that the above-belowground indirect interactions slowed down the aphid population was as well detected (Fig.5a in Chapter 4). The decreased

aphid population caused by the above-belowground interaction are likely due to the lower nymph survival (Fig. 4 in Chapter 4) and the induced formation of winged morph of aphids (Fig. 6a in Chapter 4). Wing dimorphism is a common phenomenon in many insects such as aphids and there are both wingless and winged morphs at one colony (Dixon, 1998; Müller et al., 2001). Winged aphids have lower reproduction because they need spend huge nutrient to build wings and flight muscles. However, the wingless aphids have a high fecundity to compensate the disability in flight (Dixon, 1998). The formation of winged morph in aphid colony is in response to environmental changes, reduction of host quality, and risk of predation/parasitism (Braendle et al., 2006; Brisson et al., 2010). This is consistent with our results that both above-belowground interactions and parasitoids increased the proportion of dispersal morph in aphid colony (Table 3, Fig. 6a in Chapter 4). The above-below ground interactions activated vertical migration of aphids to the top part of the host for more nutrients (Table 4, Fig. 7 in Chapter 4). Consequently, the increasing mobility of aphids would cause a 'fake crowding' within the host and thereby facilitate the formation of dispersal morphs of aphids. This could explain why a lower aphid population was observed on the plants jointly attacked by nematodes and fungi, than that on the plants infested by aphids alone.

> Above-belowground indirect interactions among nematodes, fungi and aphids interfere with biological control.

Moreover, the above-belowground interactions not only slowed down the aphid population but also impacted the biocontrol efficiency of parasitoids. We found that the parasitism rate was significantly reduced by above-belowground interactions after parasitoid release (Table 5, Fig 8 in Chapter 4). The higher mortality on aphid nymphs caused by above-belowground interactions may be due to a stronger induction and accumulation of defensive toxins that may cause the indirectly toxic effect on natural enemies via the food chain (Hopkins et al., 2009; Kos et al., 2012). Also, the alteration in volatile blends emitted by the plants attacked by both aboveground and belowground pests and the plants attacked by aphids alone, could impact the attractiveness as well to natural enemies (Shiojiri et al., 2002). It has been reported by Shiojiri et al (2002) that when cabbages are simultaneously infested by multiple herbivores, the new blend of volatiles released by the plant is less attractive to the natural enemies than that released by the plant infested by one herbivore species. To test this hypothesis, we sampled volatiles during this population dynamic experiment but the analyses of volatiles samples is still on-going. Although, above-belowground interactions could slow

down aphid population, they interfere with the biological agents as well as cause tomato yield loss (Table 6, Fig. 9a in Chapter 4).

3. At phytochemical level

Untargeted metabolomics approach was applied on tomato roots to tentatively explain the mechanisms of plant defenses induced by nematodes and the potential influence of aboveground pests on root secondary metabolites.

The obvious alteration in root metabolites was induced after tomato plants were inoculated by nematodes (Fig. 3, Fig. 7 in Chapter 5). Metabolites induced by the nematode attack and combined attacks with other aboveground pests did not significantly differ. This means the phytochemical defenses produced in roots mainly depend on the induction by nematodes. This is consistent with our biological data (Fig. 1a, 2a, 3a in Article 2). Namely, no matter when 2 species, 3 species or 4 species of pests sequentially attacked tomato plants, nematode performance was not affected by the aboveground pests. On the opposite, the presence of nematodes affect aphid and caterpillars.

The similar chemical result was also observed in cotton plants. Aboveground caterpillar *S. exigua* only induced defensive terpenoids in young leaves, but attack by root herbivores induced terpenoids in both roots and leaves (Bezemer et al., 2003). The distinction in the inducible metabolites may be due to organ specificity of defensive induction in response to pest attack. Moreover, the induced defensive compounds in roots could be transferred to shoots for the defense against aboveground pests (Kaplan et al., 2008b). Then, our next step is to analyze the leaf metabolites involving the different pest species number, locations, feeding guilds and attack sequences.

CHAPTER 7. CONCLUSIONS AND PERSPECTIVES

Overall, our studies focus on ‘plant-mediated indirect interactions’ at both individual, population and phytochemical levels and some conclusions were obtained. **Firstly**, a general relationship was firstly obtained between the pest species number involved in indirect interactions and insect performances as well as plant fitness. Namely, the herbivorous insect performances were correlated with the abundance of indirect interactions, whether pests attacked tomato plants simultaneously (positive correlation) or sequentially (negative correlation). However, performances of plant pathogenic organisms were not correlated with the number of pest species involved in plant-mediated indirect interactions. In addition, plant growth was also positively correlated with the number of pest species involved in the indirect interactions, when multiple pests attacked tomato plants in sequence. The above-belowground interactions as well depend on pest attacking sequence and pest diversity. When pests sequentially attacked tomato plants, the only belowground pest (nematodes) unidirectionally affected aboveground pests. When pests simultaneously attacked tomatoes, the interactions between aboveground and belowground depend on the pest species number on the plant. When 2 pests simultaneously attacked tomatoes, aboveground pests unidirectionally impacted nematodes. However, nematodes and aboveground pests were bidirectionally affected when 3 pests shared the host. Whether nematodes, fungi and aphids attacked tomatoes simultaneously or sequentially, the number of aphid individuals on the host was reduced by plant-mediated indirect interactions. Sublethal concentration of beta-cypermethrin accelerate the interspecific competition between the generalist aphids and specialist aphids on soybeans, and impaired the induced susceptibility of host to the interspecific aphids.

Secondly, the above-belowground interactions involving nematodes, fungi and aphids, slowed down the aphid population in long term, via reducing survival of nymphs and inducing the formation of winged aphids in the aphid colony. However, the above-belowground interactions interfere the biological control by the decreased parasitism rate of parasitoids, and cause yield loss of tomato fruits.

Finally, 316 significant metabolites were observed in the roots between control plants and pest-infested plants. However, the induction of metabolites in roots mainly relied on the nematode inoculation. Unfortunately, our research on potential phytochemical mechanisms (both on non-volatile and volatile metabolites) underlying the plant-mediated indirect interactions are still in progress. Future studies in the plant-mediated indirect interactions in a tri-trophic system may focus on:

At short term:

Phytochemical analyses are in progress, including volatiles in order to test the hypothesis of modification of the volatile blend attracting parasitoids in Chapter 5, and matrices of foliar metabolites in Chapter 6. Distinction in volatile blends between the plant infested by 3 pests (nematode, fungi and aphids), and the plant infested by aphids alone will be compared and analyzes. This may explain the potential reasons (alteration in volatile components or in volatile concentration or in others) why above-belowground interactions caused plants less attractiveness to parasitoids.

At middle term:

More kinds of experiments would be set up in order to test the hypothesis coming from our results above, such as, the difference between herbivores and pathogens response to plant defenses, impact of diverse attacking sequence on plant-mediated indirect interactions, the link between wing morphs and plant quality, olfactometry with the parasitoid to test the attractiveness to the plants with more pest infestation, etc...

For example, if we modify the attacking sequence (such as nematodes attack later than aboveground pests), which pest will have the strongest influence to the others? Is the first arriving one always the most influential? These experimental set-ups should be involved to comprehensively assess the impact of the order of pest attack on the direction of above-belowground interactions, due to the importance of attacking sequence to plant-mediated indirect interactions (Erb et al., 2011; Van Zandt and Agrawal, 2004; Viswanathan et al., 2005).

At long term:

- (1) More pest species/ more feeding guilds of pests should be involved to evaluate the relationship between pest performances and pest diversity and to validate if the relationship could be more generalized. For example, is the correlation still suitable between insect performances and pest species number, when cell-content feeders and bacteria are introduced into the interactions, or when more belowground pests are introduced into these interactions?
- (2) More focus should be on the above-belowground interactions among nematodes, fungi and aphids due to their stably suppressed effects on aphid population (McCarville et al., 2012). It may offer a new way to control aphids, if the determinant that suppressed aphid population under this above-belowground interactions could be detected. Does biological

control still work when farmers have to deal with multiple attack? It appears that it's not working for parasitoids, but would it be the same with predators?

- (3) Further analyses on metabolomics in tomato leaves and on the identification of significant molecules induced by different pests should carry on. To date, little is known in tomatoes about most secondary metabolites, such as, which is specific to a certain pest species (Howe and Jander 2008; War et al. 2012). Thereafter, a link could be built between the secondary metabolites, phytohormonal signalings and the attackers (Xia and Wishart, 2016). This may contribute to understanding the integrated strategies employed by tomatoes to defend against the biotic attackers. Moreover, some specific/significantly changed molecules observed from metabolomics analyses should be also validated its biological function on pests.

All the studies may be helpful to optimize the IPM strategy in agroecosystem.

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ANNEX 1

Establishment of a metabolomics approach and Molecular Network for the study of tomato roots (*Solanum lycopersicum*) at different maturity stages.

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Abstract

Chemical ecology promotes understanding of the origin and function of chemicals mediating interactions with and between organisms. It has strong links with metabolomics, the large-scale study of all metabolites detectable in biological samples. This paper presents the development of untargeted metabolomics approach to evaluate variability in tomato roots at two maturity stages, in order to better understanding later, the difference between healthy and infested tomato plant. 24 tomato plants (*Solanum lycopersicum*) were growth in mesocosm, dividing in two groups of 12 replicates Y and M collected respectively on the 34th and 62th days after sowing. An extraction method was developed to obtain a maximum of metabolites, and then the metabolomics footprint of the root extracts was obtained thanks to UHPLC-ESI-Q-Tof-HRMS coupling and introduced on the workflow4metabolomics platform to preprocess the data (XCMX). The information obtained by the statistical analysis *i.e.* unsupervised (PCA, HAC) and supervised (PLS-DA), and molecular networks constructed using tandem mass spectrometry (MS2) experiments were used to identify compounds that vary in tomato roots at different stages of maturity. The majority family containing in tomato roots extract are alkaloids and polyphenols, around twenty compounds are discriminating between the young (Y) and mature (M) tomato roots. This approach will be used later for study of variation between healthy and infested tomato plant.

Key words:

Solanum lycopersicum, tomato roots, untargeted metabolomics, Molecular Network, glycoalkaloids, alkaloids.

1) Introduction

Tomato (*Solanum lycopersicum*) is one of the most consumed vegetables in the world and play an important role in human diet, whether consumed fresh or as processed cooked products. In addition, tomato consumption has been associated with beneficial health properties, such as decreased serum lipid levels, reduced oxidation of low-density lipoproteins and prevention of platelet aggregation^{1,2}. Furthermore, epidemiological research has shown a correlation between increased consumption of tomato products and a decreased risk of certain diseases, including prostate cancer³. Nevertheless, tomato plants *i.e.* aerial and underground parts have to deal with many herbivorous pathogens and arthropods that differ considerably with respect to type and severity of damage inflicted, intimacy of association with the plant, and sensitivity to the various plant defense mechanisms⁴. Moreover, results affirm the importance of the inclusion of a battery of pests when evaluating the effects of novel chemical and biological agents that operate through induced plant resistance to fully assess their potential for integrated disease and insect control⁵.

Tomato defense against pathogens and arthropods is provided in part by secondary chemicals, including the synthesis of steroidal glycoalkaloids⁶, which can have lethal or sub-lethal effects on herbivorous insects⁷. A single plant can contain dozens of secondary chemicals of disparate chemical classes⁸. Thus, attraction of insects to tomato plants and other host organisms involves detection of variation of metabolome, and its understanding could help in the protection of tomato plants. However, before studying the metabolome variation in presence of insect, it is first necessary to understand the metabolic variations that are due to the age of tomato plants, *i.e.* their maturity stages. In this context, an untargeted metabolomics approach

¹ Lazarus, S. A., Garg, M. L., Int. J. Food Sci. Nutr. 2004, 55, 249–256.

² Proteggente, A. R., Pannala, A. S., Paganga, G., Van Buren, L., Wagner, E., Wiseman, S., Van De Put, F., Dacombe, C., Rice-Evans, C. A., *Free Rad. Res.* 2002, 36, 217–233.

³ Edward Giovannucci; Tomatoes, Tomato-Based Products, Lycopene, and Cancer: Review of the Epidemiologic Literature. *Journal of the National Cancer Institute*, Vol. 91, No. 4, February 17, 1999

⁴ M. J. Stout; Signal interactions in pathogen and insect attack: systemic plant-mediated interactions between pathogens and herbivores of the tomato, *Lycopersicon esculentum*. *Physiological and Molecular Plant Pathology* (1999) 54, 115±130.

⁵ M. J. Stout, A. L. Fidantsef, S. S. Duffey and R. M. Bostock; Signal interactions in pathogen and insect attack: systemic plant-mediated interactions between pathogens and herbivores of the tomato, *Lycopersicon esculentum*. *Physiological and Molecular Plant Pathology* (1999) 54, 115±130

⁶ Maxim Itkin, Ilana Rogachev, Noam Alkan, Tally Rosenberg, Sergey Malitsky, Laura Masini, Sagit Meir, Yoko Iijima, Koh Aoki, Ric de Vos, Dov Prusky, Saul Burdman, Jules Beekwilder, and Asaph Aharonia. GLYCOALKALOID METABOLISM1 Is Required for Steroidal Alkaloid Glycosylation and Prevention of Phytotoxicity in Tomato. *The Plant Cell*, Vol. 23: 4507–4525, December 2011.

⁷ Szymon Chowański, Zbigniew Adamski, Paweł Marciniak, Grzegorz Rosiński, Ender Büyükgüzel, Kemal Büyükgüzel, Patrizia Falabella, Laura Scranò, Emanuela Ventrella, Filomena Lelario and Sabino A. Bufo. Review of Bioinsecticidal Activity of Solanaceae Alkaloids. *Toxins* 2016, 8, 60; doi:10.3390/toxins8030060.

⁸ Duffey SS, Stout MJ. 1996. Antinutritive and toxic components of plant defense against insects. *Archives of Insect Biochemistry and Physiology* 32: 3-37.

was developed and applied to study phytochemical variations in tomato roots at different development stages.

Here we present a comparative untargeted metabolomics strategy based on ultra-high performance liquid chromatography – time of flight mass spectrometry high resolution with electrospray ionization (UHPLC-ESI-Q-ToF-HRMS), data processing in workflow4metabolomics (W4M) platform⁹, statistical analysis using the open source MetaboAnalyst¹⁰ and dereplication information obtained by means of Molecular Network using The Global Natural Products Social Molecular Networking (GNPS) platform¹¹.

Chemical ecology promotes understanding of the origin and function of chemicals mediating interactions with and between organisms. It has strong links with metabolomics, the large-scale study of all metabolites detectable in biological samples. Metabolomics is a growing field in which analysts seek to comprehensively analyze and compare quantities of metabolites (small molecules) in biological samples^{12,13}. Metabolomics is applied to facilitate understanding of the disease pathogenesis¹⁴, to study the effects of diet and drug interactions¹⁵, to identify biomarkers¹⁶, and to discover natural products and drugs¹⁷.

Mass spectrometry is often the analytical technology of choice for metabolomics research because of its unparalleled ability to analyze the ability to detect metabolites present at low concentrations¹⁸. Tandem mass spectrometry (MS/MS) experiments often generate millions of spectra that can be used to identify thousands compounds in complex samples. Analyzing such large data sets poses a computational challenge. In this context, we created MS/MS-Clustering algorithm for the measure of spectral similarity and construction of cluster representatives¹⁹. MS/MS spectra can be collected directly from complex mixtures or with the

⁹ Yann Guitton; Create, run, share, publish, and reference your LC–MS, FIA–MS, GC–MS, and NMR data analysis workflows with the Workflow4Metabolomics 3.0 Galaxy online infrastructure for metabolomics. *International Journal of Biochemistry and Cell Biology* 93 (2017) 89–101.

¹⁰ Jianguo Xia ; MetaboAnalyst: a web server for metabolomic data analysis and interpretation. *Nucleic Acids Research*, 2009, Vol. 37, Web Server issue.

¹¹ Robert A. Quinn; Molecular Networking As a Drug Discovery, Drug Metabolism, and Precision Medicine Strategy. *Trends in Pharmacological Sciences*, February 2017, Vol. 38, No. 2.

¹² F. Matsuda, Technical challenges in mass spectrometry-based metabolomics, *Mass Spectrom (Tokyo)* 5 (2) (2016) S0052.

¹³ M.S. Monteiro, M. Carvalho, M.L. Bastos, P. Guedes de Pinho, Metabolomics analysis for biomarker discovery: advances and challenges, *Curr. Med. Chem.* 20 (2) (2013) 257e271.

¹⁴ X. Li, A. Zhang, H. Sun, Z. Liu, T. Zhang, S. Qiu, L. Liu, X. Wang, Metabolomic characterization and pathway analysis of berberine protects against prostate cancer, *Oncotarget* (2017).

¹⁵ D.S. Wishart, Applications of metabolomics in drug discovery and development, *Drugs R* 9 (5) (2008) 307e322.

¹⁶ B.L. Ackermann, J.E. Hale, K.L. Duffin, The role of mass spectrometry in biomarker discovery and development, *Curr. Drug Metabol.* 7 (5) (2006) 525e539.

¹⁷ B.L. Ackermann, J.E. Hale, K.L. Duffin, The role of mass spectrometry in biomarker discovery and development, *Curr. Drug Metabol.* 7 (5) (2006) 525e539.

¹⁸ J. Boccard, J.L. Veuthey, S. Rudaz, Knowledge discovery in metabolomics: an overview of MS data handling, *J. Separ. Sci.* 33 (3) (2010) 290e304.

¹⁹ Frank et al., *journal of proteome research* 2008, 7, 113-122.

added separation provided by UHPLC²⁰. In phytochemical, the compounds belonging to the same family can have similar MS/MS spectra because molecules with similar structures fragment into many of the same substructures, it permit grouping of molecules according to their belonging to a molecular family. The GNPS platform can be used for measure this spectral similarity and construct a Molecular Network representing a sample mapping of extract chemical composition. The Molecular Network approach has several utilities *e.g.* visualizing of structures relationship among unknown metabolites, identification of known compounds by searching public MS libraries, and comparative study among plants species²¹.

2) Material and Methods

2.1 Samples preparation

2.1.1 Chemicals

All the solvents used for the plant extraction and UHPLC-HRMS analysis were HPLC grade: ethanol and methanol were purchased from VWR (Fontenay-sous-Bois, France). Acetonitrile from SDS Carlo Erba (Val de Reuil, France) used for UHPLC-HRMS analysis was HPLC PLUS gradient grade. The ultrapure water was produced with a PurelabFlex system from Veolia (Wissous, France). Gallic acid standard ($\geq 98\%$) was purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). Alpha-tomatine ($\geq 98\%$) and digitonin ($\geq 98\%$) were purchased from Extrasynthese (Genay, France). The stock solution of alpha-tomatine was prepared by dissolving pure powder in ethanol at 1 mg/mL.

2.1.2 Mesocosm experiments

Tomato plants, *Solanum lycopersicum*, Solanaceae; cultivar “San Marzano Nano”, were grown in mesocosms Figure 1 ($23\pm 0.3^\circ\text{C}$ at daytime, $18\pm 0.2^\circ\text{C}$ at night, RH: $66\pm 1\%$, irradiance: $663\pm 48 \text{ W/m}^2$). The one-week-old seedlings were separated by pots and planted in a mixture with soil and perlite and supplied with standard nutrient solution (pH=6, electrical conductivity (EC) = 1.6 mS/cm , ion concentration: 620 mg/L NO_3^- , 11 mg/L NH_4^+ , $170 \text{ mg/L H}_2\text{PO}_4^-$, $115 \text{ mg/L SO}_4^{2-}$, 180 mg/L K^+ , 212 mg/L Ca^{2+} , 12 mg/L Mg^{2+}). On the 34th day after sowing (tomato plants with 3 full leaves), tomato roots were sampled and stored in -80°C as young stage samples (n=12 replicates *i.e.* 12 plants). In the same way, roots were collected from 12 plants as old-stage samples (blooming plants with 9 full leaves) on the 62nd day and also stored in -80°C . All the samples were lyophilized (Serail RP2V) for 48 hours to remove moisture. Then,

²⁰ Sedio et al., Mass spectrometry metabolomics.

²¹ Wang et al., 2016

they were ground with a ball mill (Retsch MM301) by shaking (30/s) with 2 metal balls with a diameter of 2cm for 2 minutes. The root powder was transferred to 2 ml Eppendorf tubes and stored in -80°C until the next step of metabolite extraction.



Figure 1: Tomato plants grown in mesocosm

2.1.3 Extraction

Plant matrix 5 mg was introduced into 400 μ L of methanol/water (75/25) acidified with 0.1 % formic acid. Digitonin (25 ng/ μ L) and gallic acid (50 ng/ μ L) were introduced in the extraction solvent as internal standards. The extraction was assisted by ultrasound (50 Hz) during 20 minutes. Then, the 24 samples were centrifuged during 10 minutes at 9800 rpm and the supernatants was transferred to vials for analysis.

Two vials containing respectively young and mature samples pooled were prepared. Young pool contains 100 μ L of each of the twelve young replicates and mature pool is a mix of twelve mature replicates (100 μ L each one).

2.2 UHPLC-ESI-Q-ToF-HRMS analysis

Chromatographic analysis was performed using an Ultimate 3000 RSLC system equipped with a binary pump, an autosampler and a thermostated column compartement (Dionex, Germering, Germany). Compounds of tomato roots were separated on a Luna omega C18 column (150 x 2.1 mm; 1.6 μ m, Phenomenex) at 40°C. The mobile phase at a flow rate of 500 μ L/min was composed of water + 0.1% formic acid (A) and acetonitrile + 0.08 % formic acid (B); the gradient program was as follows: 98% A, 2% B at initial time, 73.6% A, 26.5% B at 4.8 min, 63.8% A, 36.2% B at 8.75 min, 35% A, 65% B at 10.6 min, 10% A, 90% B at 11.1 min to 12.6 min, then the column was reequilibrated under initial conditions during 3 min. The samples were analyzed randomly, injection volume was 1 μ L.

MS experiments were performed on a maXis UHR-Q-TOF mass spectrometer (Bruker, Bremen, Germany) in positive and negative electrospray ionization (ESI) modes. Capillary voltage was set at 4.5 kV in positive mode and 4.0 kV in negative one. The flows of nebulising

and drying gas (nitrogen) were respectively set at 2.0 bar and 9 L/min and drying gas was heated at 200 °C. The analysis was made with an acquisition frequency of 2.5 Hz, the mass scan range was set from m/z 50 to 1550. Data were processed using DataAnalysis 4.4; the resulting fingerprints were displayed as base peak chromatograms (BPCs) to be compared. MS/MS experiments were carried out using data dependent acquisition (DDA) mode. The collision energy was limited to 12 eV with the exception of molecules in the range m/z 400-450 and compounds beyond m/z 800 fragmented respectively to 50 eV and 70 eV.

Compounds were identified using combination of the accurate mass, isotope ratio, MS/MS fragmentation and GNPS spectral libraries. DataAnalysis 4.4 Bruker software was also used to analyze and identify the compounds using MS and MS/MS spectra.

2.3 Data preprocessing

Bruker files acquired in positive mode were converted into mzXML format using CompassXport 3.0.13 utility (Bruker). Preprocessing of the data were conducted on the online and freely available workflow4metabolomics (W4M) platform <http://workflow4metabolomics.org>, to obtain a final matrix with samples in lines, and the compounds in columns. This process includes several steps such as peak picking, alignment, integration to have matrix ready for statistical analysis or “post processing”. This work was done using the implementation of the XCMS software in W4M ²². The process used for this study provided 4 tools that must be set to build of the comprehensive workflows for LC-HRMS data analysis Table 1.

| Tools | Parameters |
|----------------|---|
| xcms.xcmsSet | Scanrange50-708 S/N20 centWave method |
| xcms.group | Bandwidth5 |
| xcms.rector | Obiwarpmethod |
| xcms.fillpeaks | Fillingmethodchrom |

Table 1: The applied workflow to realize the peakpiking with workflow4metabolomics (XCMS algorithm)

²²Engel J. Breaking with trends in pre-processing?. TrAC Trends in Analytical Chemistry. Volume 50, October 2013, Pages 96-106

The first tool is used to filter and identify peaks by extract ion chromatograms, apply model peak matched filter, then, identify and integrate peak areas. The second tool is make to groups together the peaks that represents the same analyte after peaks identification. The third one is applied to retention time correction, by identify groups for use as standards, calculate retention time deviations and align peaks using non-linear warping. The last step of data-preprocessing is applied to integrate peaks areas of all of analysis and create matrix containing variables with their area in each sample²³

The workflow (supplementary material) was created and applied to one compress file (with 7-Zip File Manager) containing the 24 mzXML data (12 young and 12 mature samples). This file was introduced in the W4M platform using Cyberduck. In addition to the analyzes, one sample metadata file (.txt) was inserted into the W4M platform to have the information about the samples (sample name, class, sample type, batch, injection order).

2.4 Statistical analysis

MetaboAnalyst 4.0 was employed for the analysis of data. The univariate (Volcano Plot) and multivariate analysis, including unsupervised (PCA, HAC) and supervised (PLS-DA) were used to identify differences among groups.

The matrix, generated by W4M, including 72 variables and 24 samples without missing value (imputed by intensity values) is used to make comparative analyzes between young and mature groups. It is inserted in MetaboAnalyst 4.0, the auto-scaling algorithm was used to normalize the variable intensity. The hierarchical clustering is obtained using MetaboAnalyst 4.0 and the following options: Euclidean distance measure and ward clustering algorithm.

2.5 Molecular Network

The Molecular Network is generated using the online workflow Global Natural Products Social Molecular Networking (GNPS, <http://gnps.ucsd.edu> ²⁴). First, the AutoMS(n) algorithm of DataAnalysis 4.4 software was applied to profile spectra of analyzes after background subtraction, to generate the peak-list of tomato roots compounds. The retention time window is 0.3 min, intensity threshold TIC (AllMSn) fixed to 50 for positive mode (ESI⁺), and maximum number of compounds at 7500. The peak-list of young and mature analyzes were exported in MGF format. The most abundant ions were exported: 10 non-deconvoluted

²³ Anal. Chem. 2006, 78, 779-787

²⁴ Wang et al. ; Sharing and community curation of mass spectrometry data with Global Natural Products Social Molecular Networking ; 2016.

(intensity threshold 100). This format expresses the MS/MS spectra into a peak-list containing all the information about the analysis: precursor ions (m/z) with their retention time, fragment ions (m/z , intensities, charge). Then, the peak-lists of young and mature groups were sent to the GNPS platform. The data were afterwards processed with a parent mass and fragment ion both with tolerance of 0.02 Da to generate consensus spectra representing putative unique molecular structure. Edges were formed for spectral matches with cosine score ≥ 0.65 and matched peaks ≥ 4 , furthermore, edges between two nodes were kept in the network if and only if each of the nodes appeared in each other's respective top 10 most similar nodes.

In addition, for the identification of compounds, the spectra in network were searched against the GNPS's spectral libraries in the same manner as input data using the following parameters: score threshold 0.65, minimum matched peaks 6, maximum analog search mass difference 100. Finally, Molecular Network was visualized using Cytoscape 3.5.1 software.

3) Results and discussion

An untargeted metabolomics approach was taken to proudly evaluate and characterize compounds differences between young and mature tomato roots. It is important to comprehensively understand the phytochemical differences between young and mature tomato roots and know the variations that are due to roots maturity in order to better detect phytochemical variation during plant infestation. The design of a metabolomics study can be divided in four basic steps: sample collection, metabolite extraction, chromatographic and spectroscopic analyses, and finally data treatment including statistics and metabolite identification²⁵. Extraction and chromatographic separation are essential steps to get the widest range of features. The statistical analyses allows comparing the spectral signature of tomato roots and detect the differences between metabolome.

3.1 Method development and MS optimization

3.1.1 Sample preparation conditions

In untargeted plant metabolomics, the extraction procedure must be able to solubilize compounds displaying a wide range of physicochemical properties and may be found in varying concentration. Several studies have suggested that chemical diversity is increased when multiple solvents are used during extraction process. Best solvent

²⁵ Metabolomics: What You See is What You Extract. *Phytochem. Anal.* 2014,25, 289–290

systems are usually made with mix of alcohol (e.g. ethanol, methanol) and water^{26,27}. Furthermore, previous metabolomics work^{28,29} on tomato used pure methanol or 75% methanol for unselective extraction of tomato compounds. Within this context, we first evaluate different proportion of methanol/water (MeOH/H₂O) (100/0), (75/25), (50/50), (0/100) in order to maximize number of extracted compounds. Comparison of chromatographic profiles allowed us to choose the ratio 75/25 (MeOH/H₂O). This solvent was able to extract polar and semi-polar compounds. Addition of formic acid enabled extraction of glycoalkaloids, typical compounds of tomato species³⁰. Finally, two internal standards were added to extraction solvent, gallic acid and digitonin. Both are absent from root extracts and they do not induce co-elution. The former was selected according to the presence of phenolic compounds while the latter, a saponin-like molecule, was chosen to mimic glycoalkaloid behavior.

3.1.2 UHPLC-ESI-Q-ToF-HRMS analysis

Considering the polarity of extracted metabolites, water and acetonitrile were chosen to eluate the compounds, both with formic acid because the majority of compounds are left hanging on the C18 column when mobile phases without formic acid were tested. The optimized chromatographic conditions were a compromise between peak resolution and total run time, resulting in separation of different molecules classes.

MS experiments were performed in ESI given the polarity of the molecules, the positive and negative modes were tested in order to detect the most compounds. ESI⁺ mode was chosen to analyze the tomato roots samples since it enabled to detect some compounds not responding in the negative mode, endless the MS spectra with ESI⁺ of glycoalkaloid are easier for interpretation, with pseudo-molecular ion and fewer adduct. However, the MS spectra obtained using ESI⁻ have helped identification of some compounds. In regards to frequency acquisition, several were tested: from 1 to 5 Hz to improve the peak resolution and get enough data points

²⁶ Evaluating solvent extraction systems using metabolomics approaches RSCAdv.,2014,4,26325–26334.

²⁷ Plant metabolomics - meeting the analytical challenges of comprehensive metabolite analysis. BRIEFINGS IN FUNCTIONAL GENOMICS. VOL 9. NO 2. 139-148.

²⁸ Metabolite profiling and quantification of phenolic compounds in methanol extracts of tomato fruit. Phytochemistry 71 (2010) 1848–1864.

²⁹ A Liquid Chromatography-Mass Spectrometry-Based Metabolome Database for Tomato. Plant Physiol. Vol. 141, 2006.

³⁰ Chemistry and Anticarcinogenic Mechanisms of Glycoalkaloids Produced by Eggplants, Potatoes, and Tomatoes. J. Agric. Food Chem. 2015, 63, 3323–3337.

to define one peak for deconvolution by W4M algorithm. The frequency acquisition of 2.5 Hz was selected since it allowed optimal resolution and signal-to-noise ratio.

Finally, several collision energies were tested for MS/MS experiments to find the optimal ones which allowed to fragment the rigid structures while having correct spectra for molecules that fragment easily. An energy of 12 eV was chosen to fragment most compounds, 50 eV to fragment rigid alkaloids and 70 eV to break the glycoalkaloids with genin more rigid.

Base Peak Chromatograms (BPC) obtained using UHPLC-HRMS in positive mode of young and mature pools are presented in Figure 2. This result shows that most of compounds appear present in both types of sample, the differences are mainly manifested by differences in intensity of some compounds being more intense in young and others in mature group.

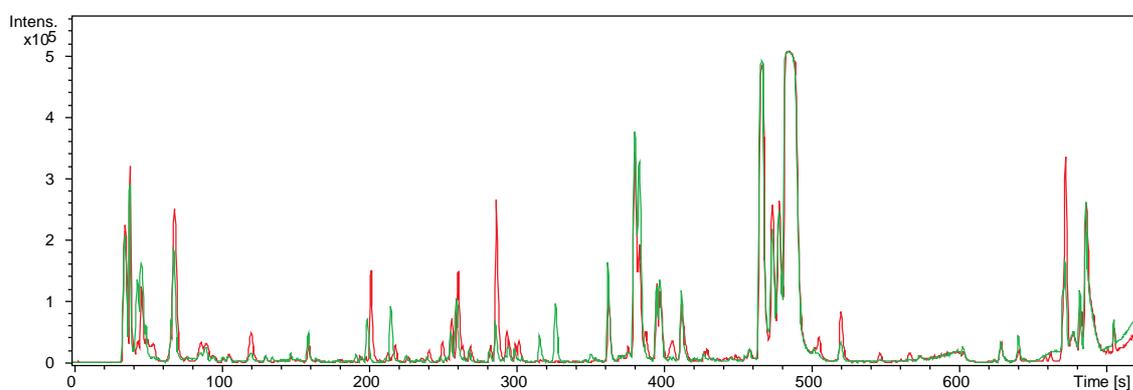


Figure 2: The Base Peak Chromatogram of young (red) and mature (green) tomato roots extracts obtained using UHPLC-HRMS in positive mode (ESI+). Separation was performed on Luna omega C18 column (150 x 2.1 mm; 1.6 μ m, Phenomenex) with gradient elution of water and acetonitrile both acidified with 0.1% of formic acid.

3.2 Data preprocessing

The matrix of all the analysis contains line X: individu corresponding to the different samples, column: variable corresponding to each detected molecule named by the most intense ion (m/z) with retention time in seconds, and response line Y: intensity of the most intense ion.

A total of 855 features were generated from LC-HRMS analyzes by W4M, resulting in history of 27 files (total size: 71.79 MB). The large variable number could provide instability to the statistical model, which implies irrelevant statistical results. To solve this problem, statistical tests were combined to have a smaller dataset from the initial matrix. The starting matrix was introduced in the online software MetaboAnalyst 4.0; the variables were centered and reduced. Then, the PCA and HAC were used to visualize the spontaneous groups separation, the PLS-DA analysis was applied to obtain the VIP score of variables (variable important on the projection) allows to choose the compounds. Only 72 variables (number of variable = 3 x number of individus) with higher VIP score according to component 1 were chosen to create a sub-matrix containing the most relevant variables.

The short matrix of 72 variables and 24 individus was extracted from the starting matrix (72 most important on the projection: highest VIP score) and used for statistical analysis on the MetaboAnalyst 4.0 software to determinate biomarkers discriminate young and mature tomato roots.

3.3 Statistical analysis

Unsupervised principal component analysis (PCA) and hierarchical clustering analysis (HCA) approaches were first used to visualize groups, trends, and outliers among the observations Figure 3. The PCA score plot showed a distinct separation across the two groups, suggesting differences in metabolic profile when tomato plants are collected with a lag of 22 days. The first 2 components capture 85% of the total variation. The young and mature clusters are well discriminated according to component 1 (79%). These PCA shows the absence of outlier, and allows separation of young and mature groups. The second figure (B) is the dendrogram representing hierarchical clustering of the twenty four samples of tomato roots. The horizontal axis of dendrogram represents the distance or dissimilarity between clusters, the vertical axis the objects and clusters. This presentation shows two clusters as two branches occur at about the same horizontal distance: young (red) and mature (green) groups. The intra-group dispersion of mature group observed on the PCA would be due to young 1 and 2 which have a greater distance on the dendrogram compared to the other individus.

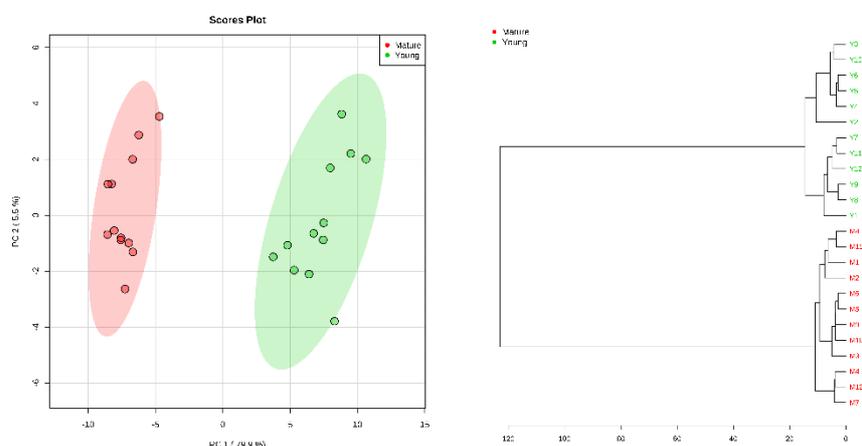
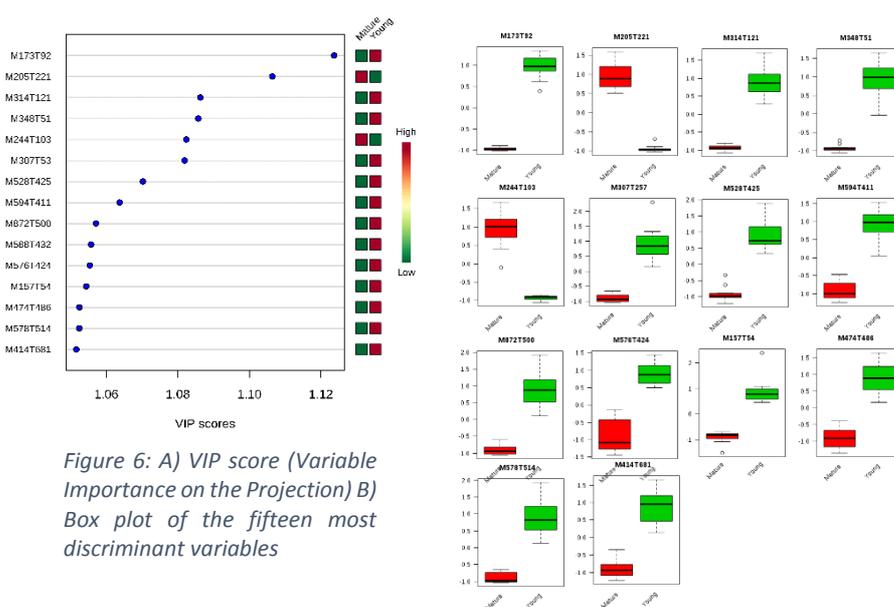


Figure 3: Unsupervised multivariate analysis A) Principal Component Analysis of young (red) and mature (green) tomato roots, and B) dendrogram of hierarchical clustering result

The unsupervised analyses show the good separation of young and mature tomato roots with 85% of information (component 1 and 2). The dendrogram show the distance between all samples and the responsible individus of intragroup dispersion. However, to found the compounds which discriminate the groups and are responsible for the separation of tomato

Figure 6 shows VIP score and Box-plot of compounds discriminating the young and mature groups. The VIP score obtained from the PLS-DA allows to obtain the most relevant variables, those which are responsible for the separation of the roots groups according the component contains the most information. In this study, VIP score = 1 was defined to limit the selection of variables, their discrimination of roots groups is important when a VIP score is high. This test enable also to know the distribution of the variable in the different groups, the square is red when it is more intense and green if the variable is present in low intensity. Finally, the correlation between the variables is analyzed to eliminate those that represents the same compound and to keep only the biomarkers.

The box plot of biomarkers are given in Figure 6. The average intensity of the group is represented by the thick line, the quartile Q1 and quartile Q3 are the box limits, and mustaches are extreme values. Those biomarkers were subsequently identified. This result shows that the majority of compounds are more intense in young tomato roots, they are molecules that decrease after growth.



The statistical biomarkers were thereafter compared to chromatographic peaks who vary considerably between younger and mature groups. The aim of this step is to compare biomarkers with the chromatogram to find out if the statistical biomarkers are the same as the differential peaks on the BPC. The approach is to analyze the BCP of young and mature groups to identify the peaks that vary considerably between analyzes, and to check if these compounds that discriminate groups were highlighted by statistical analysis.

The Figure 7 associate the information obtained in Figure 2 and Figure 6, it shows the chromatographic peaks belonging to the statistical biomarkers, noted in the order of VIP score (the biomarker 1 is the compound that varies the most between the two groups, it has the highest VIP score). A variation of intensities of these compounds is observed, confirming that these biomarkers vary significantly after maturation. This comparison shows that statistical biomarkers have a different polarity, they are distributed over the entire chromatogram, some are abundant and others less so, they would therefore belong to different molecular families. This result allows to observe the chromatographic differences, and confirms that the differential peaks were highlighted by the statistical analyses, therefore, validates it.

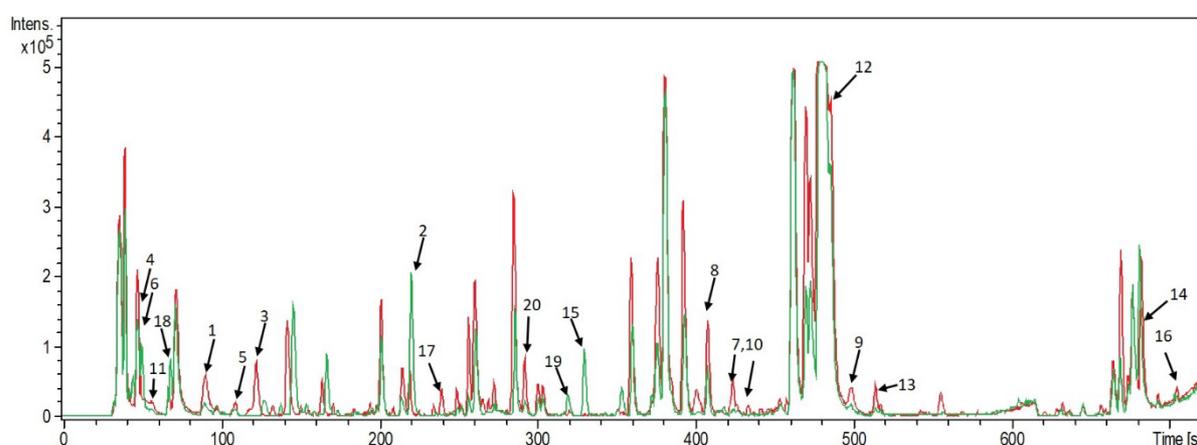


Figure 7: Indication of twenty statistical biomarkers on the base peak chromatogram of young (red) and mature (green) tomato roots. The numbering of statistical biomarkers is proportional to the VIP score.

3.4 Molecular Network

Generation of Molecular Network was based on mass spectrometry analysis of the crude extract. MS/MS spectra of the compounds were compared pairwise to find similarities in fragmentation pathways, *i.e.* the same fragment ions or similar neutral losses. To obtain suitable MS/MS spectra with a convenient number of fragment ions, several collision energies were tested for the MS/MS experiments to find the optimal ones that fragmented the rigid structures while producing good spectra for molecules that fragment more easily. Molecules presenting similar fragmentation patterns were grouped in the same cluster while the molecules with different MS/MS spectra were not associated^{31,32}.

³¹ Frank, A. M., Bandeira, N., Shen, Z., Tanner, S., Briggs, S. P., Smith, R. D., & Pevzner, P. A. (2008). Clustering millions of tandem mass spectra. *Journal of Proteome Research*, 7(1), 113- 122.

<https://doi.org/10.1021/pr070361e>.

³² Wang, M., Carver, J. J., Phelan, V. V., Sanchez, L. M., Garg, N., Peng, Y., Bandeira, N. (2016). Sharing and community curation of mass spectrometry data with Global Natural Products Social Molecular Networking. *Nature Biotechnology*, 34(8), 828- 837. <https://doi.org/10.1038/nbt.3597>.

This approach was chosen to facilitate the identification of biomarkers that differentiate between young and mature tomato roots. Indeed, the annotation of molecules already described in the literature and standards (Table 2) on the Molecular Network makes it possible to identify clusters, *i.e.* molecular families, which quickly indicates the nature of biomarker according to it cluster. In addition, the submission of mass spectra to GNPS libraries allows the identification of known compounds; therefore, it is possible to focus only on the identification of unknown compounds by their MS/MS spectra using DataAnalysis software.

The Molecular Network of tomato roots *i.e.* BI-CK (red) and CK (green) groups visualized using Cytoscape 3.5.1 software is presented in Figure 8. Every node is indicating a particular ion detected by MS/MS *i.e.* molecular, fragment or adduct ion, and the edges are representing relationship between different nodes.

On the right, the global Molecular Network containing clusters linking nodes with at least four common ions and/or same neutral losses, and solitary nodes that are not related to clusters because they have different chemical structures or have less than four ions in common with other nodes. On the left, a zoom on four identified clusters *e.g.* alkaloid clusters, representing the majority molecular family contained in tomato roots, and small cluster gathering the amino acids of tomato roots. This figure shows alkaloid family separated into three clusters: alkaloids, glycoalkaloids with genin A and glycoalkaloids with genin B. This division is due to the fragmentation of glycoalkaloid genins, which produces different substructures.

The nodes surrounded in blue present the annotated compounds described in Table 2. The nodes surrounded in yellow present the compounds highlighted by statistical analysis *e.g.* biomarkers, they have higher VIP score namely they vary in tomato roots after growth.

No cluster of flavonoid and phenolamide was observed on Molecular Network despite their presence in tomato roots because their MS/MS spectra contains less than 4 fragment ions, therefore they are solitary in Molecular Network.

| Rt (min) | Measured m/z [M+H] ⁺ | Molecular formula [M] | Calculated m/z [M+H] ⁺ | Error (ppm) | Compound assignment | References |
|----------|-----------------------------------|--|-------------------------------------|-------------|-----------------------------------|-------------------|
| 2.68 | 166.0861 | C ₉ H ₁₁ NO ₂ | 166.0863 | 1.1 | Phenylalanine | [] |
| 10.30 | 432.3469 | C ₂₇ H ₄₅ NO ₃ | 432.3472 | 0.8 | 3-Deamino-3β-hydroxysolanocapsine | [³³] |
| 11.18 | 416.3518 | C ₂₇ H ₄₅ NO ₂ | 416.3532 | 1.2 | Tomatidine | [6] |
| 6.43 | 1034.5532 | C ₅₀ H ₈₃ NO ₂₁ | 1034.5530 | -0.1 | Alpha-tomatine | Stantard |
| 6.04 | 1050.5460 | C ₅₀ H ₈₃ NO ₂₂ | 1050.5479 | 1.9 | Lycoperside H | [6] |
| 6.17 | 1048.5316 | C ₅₀ H ₈₁ NO ₂₂ | 1048.5323 | 0.7 | Hydroxy-dehydrotomatine | [6] |

³³ 3-Desamino-3α-hydroxysolanocapsine—a steroidal alkaloid from *Solanum aculeatum*. *Phytochemistry* Volume 22, Issue 9, 1983, Pages 2099-2100.

Table 2: Annotated compounds on the Molecular Network of tomato root ethanolic extract

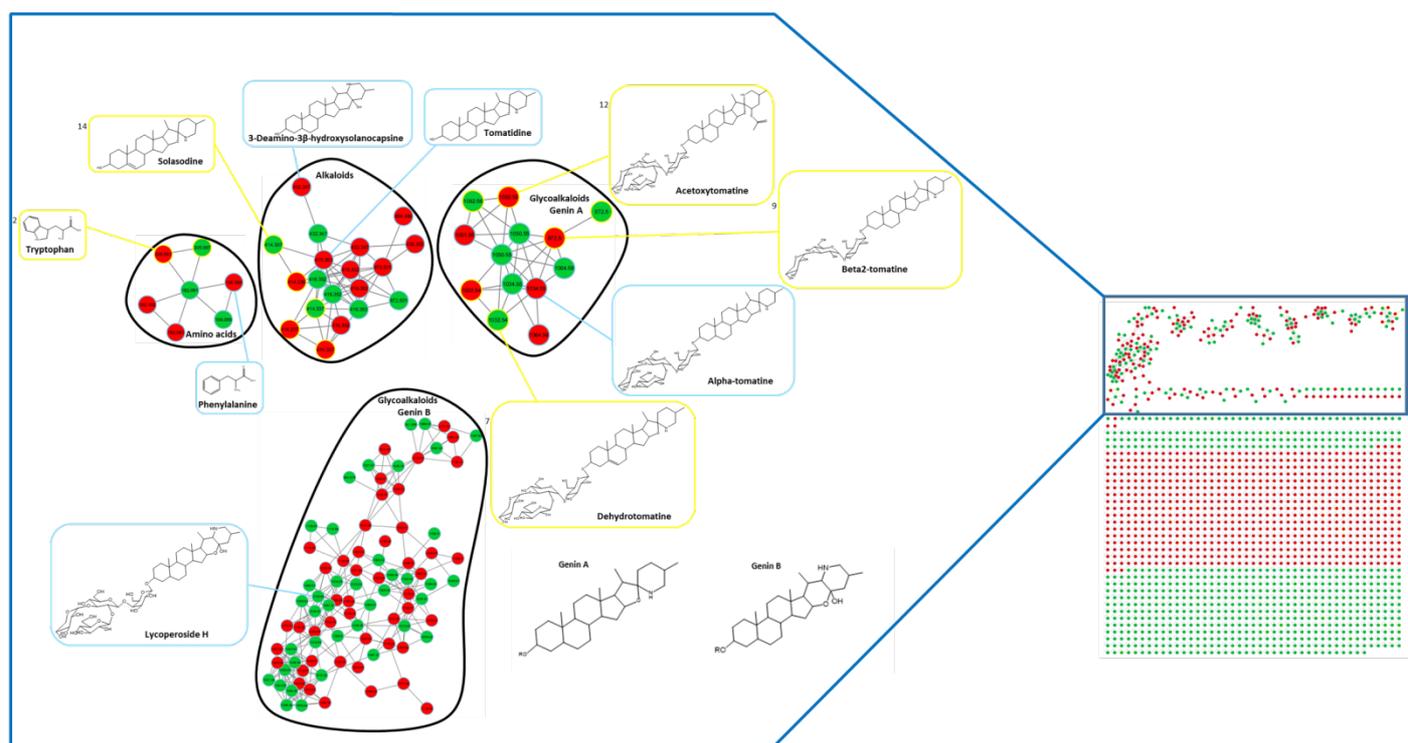


Figure 8: young (red) and mature (green) tomato roots Molecular Network; obtained using GNPS platform and visualized with Cytoscape 3.5.1 software.

Molecular Network generation provides a global view of the chemical composition of tomato roots. It highlights the majority molecular family contained in tomato roots *i.e.* alkaloids. In addition, the Molecular Network makes it possible to target families that differentiate between young and mature tomato roots and facilitates biomarkers identification.

3.4.2 Biomarkers characterization

Identification of compounds reported to be differentiate young and mature tomato roots was done using on one side the dereplication information, obtained by means of Molecular Network that allows the quick identification of known substances present in GNPS spectral libraries and/or the belonging to a molecular family. On the other using DataAnalysis software that propose chemical formula which can correspond to ions of MS spectra, with specifying the difference between the theoretical and experimental m/z calculated as Δ ppm, and comparing the isotopic profile of the experimental and theoretical molecular ion calculated as mSigma.

In short, the masses of biomarkers were searched using Extracted Ion Chromatogram (EIC) in the Base Peak Chromatogram (BCP). The mass tolerance was set at 4 ppm, or less than 7 ppm in case of fragments and small masses. Elements C, H, O to infinite number and N (0-3) were selected for calculation of elemental composition and only consistent chemical formulae were taken into account. In addition, chromatographic characteristics and HRMS/MS data as well

as bibliographic information were employed for identification. The adduct ion $[M+Na]^+$ especially observed in case of glycoalkaloids confirmed the proposed formula for the $[M + H]^+$ ion. The possible molecular formulae for each selected peak were searched in public databases *e.g.* PubChem, ChEMBL, ChEBI to report known natural products and/or eliminate non-referenced formulas. In addition, some compounds were unambiguously identified by comparing their retention time and spectral data with those of the reference standards. Finally, knowledge of the molecular family of biomarkers through the Molecular Network makes it possible to choose and confirm chemical formulae when several are coherent.

Table 3 summarizes chromatographic, mass and MS/MS fragmentation characteristics of the statistical biomarkers (higher VIP score) with their proposed chemical formulae and structures.

1) Conclusion

We have developed an effective untargeted metabolomics approach for the study of chemical variation of tomato roots extract young and mature.

Acknowledgments

References

Table Legends

Sublethal and hormesis effects of beta-cypermethrin on the biology, life table parameters and reproductive potential of soybean aphid *Aphis glycines*

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Accepted: 16 June 2017 / Published online: 6 July 2017
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Abstract Beta-cypermethrin has long been recommended as an effective pesticide to control the soybean aphid, *Aphis glycines* Matsumura, a serious pest in soybean crops. Besides acute toxicity, it leads to changes in life history traits of *A. glycines*, notably its reproductive potential. This study has assessed the effects of five sublethal concentrations (0.625, 1.25, 2.5, 5 and 10 µg/L) of beta-cypermethrin on different life history traits of *A. glycines*. Exposure to these concentrations caused shorter oviposition period and reduced adult longevity. The strongest stimulatory effect on aphid reproduction was achieved when exposed to a higher sublethal beta-cypermethrin concentration (5 µg/L). Net reproduction rate (R_0), intrinsic rate of increase (r_m) and finite rate of increase (λ) were significantly higher than that of the control, increasing by 20.58, 4.89 and 2.06%, respectively. We found no significant difference in mean generation time (T) between the treatment of 5 µg/L beta-cypermethrin and the control. However, when the concentration increased to 10 µg/L, the reproduction behavior was restrained and the mean generation time (T) was shortened, resulting in significant decrease in R_0 and T by

16.58 and 3.83%, respectively. In conclusion, a sublethal concentration (5 µg/L) of beta-cypermethrin triggered the strongest hormesis on *A. glycines*, thus providing valuable knowledge on the sublethal effects of this insecticide on soybean aphids. Hormesis may be one of the mechanisms underlying pest resurgences, and better knowledge would enable a more effective use of insecticides in Integrated Pest Management programs.

Keywords Sublethal concentration · Biological traits · Demographic parameter · Integrated Pest Management · Pest resurgence

Introduction

Sublethal effects are defined as physiological and/or behavioral effects on individuals that survive exposure to a toxic compound at sublethal doses (Desneux et al. 2007). These subtle effects can be negative on behavioral and physiological traits of arthropods, including longevity, development rate, fecundity, sex ratio, feeding activity, food searching and oviposition (Desneux et al. 2004; He et al. 2013; Chen et al. 2015; Fekri et al. 2016; Yao et al. 2015; Rix et al. 2016). Such modifications may have significant influence on the demography of exposed populations (Stark and Banks 2003; Desneux et al. 2007). Moreover, it has been reported that in a growing number of arthropod species, pesticide-induced hormesis may account for pest resurgence (Cordeiro et al. 2013; Guedes et al. 2016).

Life table parameters are effective indexes that can be used to assess the overall sublethal effects at the population level (Stark and Banks 2003; Biondi et al. 2013).

Yanyan Qu and Da Xiao contributed equally to this work.

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The survival, development and reproduction of the insect individuals exposed to increasing concentrations of a toxicant can be observed and recorded over their life span. Net reproductive rate (R_0 ; the population growth rate per generation with regard to the number of female offspring produced per female), intrinsic rate of increase (r_m : the ability of a population to increase logarithmically in an unlimited environment), finite rate of increase (λ : the factor by which a population multiplies) and mean generation time (T : the average interval separating births from one generation to the next) are four main indexes calculated in life table experiments (Carey 1993). The intrinsic rate of increase (r_m) is a key parameter, and represents the ability of a population increasing logarithmically. It has been proposed as a more effective parameter to predict the development of population in an unlimited environment (Birch 2007). In addition to assessment of individual-level effects of pesticides, studying the impact on demographic parameters e.g. the intrinsic rate of increase, is proposed as the more reliable assessment on the toxic effect of insecticides (Forbes and Calow 1999, Stark and Banks 2003, Desneux et al. 2006a; 2007).

The soybean aphid, *Aphis glycines* Matsumura (Hemiptera: Aphididae) is a phytophagous insect that feeds on soybean phloem and causes significant damage to soybean, *Glycine max* (L.). The main injuries of the plants caused by the *A. glycines* are leaf curling, premature plant development, stunted growth, fewer pod set and smaller seed size (Wang et al. 1996; Beckendorf et al. 2008). In addition, this pest can transmit numerous plant viruses, including soybean mosaic virus, bean yellow mosaic virus, tobacco vein mottling virus and tobacco etch virus (Hill et al. 2001), which can decrease seed quality and cause yield losses (Ragsdale et al. 2011; Damsteegt et al. 2011). *Aphis glycines* is native to Asia but was detected in North America in 2000, thereafter spreading to 30 states in the US and 3 provinces in Canada by 2009; and became a major pest on soybean crops in North America (Ragsdale et al. 2011). Given the potential for economically significant damage, several control strategies have been experimented and employed against this pest. Although environmentally-friendly control tactics, such as the use of resistant plant varieties (Li et al. 2004; Mensah et al. 2005) and the use of generalist and specialized natural enemies for biological control (Desneux et al. 2006b; 2009; 2012; Miao et al. 2007; Lundgren et al. 2009) have been promoted through biological control programs, chemical control measures still play a basic and important role in Integrated Pest Management (IPM) programs against this pest (Ragsdale et al. 2011).

Beta-cypermethrin belongs to the pyrethroid family. It has a broad insecticide spectrum and a wide application in the control of pests (Gao et al. 2008). In addition, sublethal

effects of beta-cypermethrin have been recently reported for various insect pests. For example, the pupation rate, emergence rate and oviposition period of *Plutella xylostella* exposed to sublethal concentration (LC_{10}) of beta-cypermethrin were significantly decreased. However, fecundity of treated *P. xylostella* significantly increased (Han et al. 2011). Moreover, sublethal concentrations of beta-cypermethrin (LC_{10} and LC_{15}) reduced longevity and fecundity of *Acyrtosiphon pisum* adults (Wang et al. 2014).

Pests may be exposed to sublethal concentrations of pesticides, due to pesticide degradation in fields after initial applications, as well as varying distribution at the time of applications (Desneux et al. 2005). Such an exposure may prompt unintended effects on pests, such as hormesis, and may lead to pest outbreaks (Guedes et al. 2016). Hormesis is a dose-response relationship for a single endpoint whose characteristic is the reversed response between low and high dose of a stressor (Kendig et al. 2010). Research on such sublethal effects of pesticides on pests is important for optimizing pesticide-based IPM (Planes et al. 2013; Guedes et al. 2016). However, potential sublethal effects of beta-cypermethrin on the demographic parameters of *A. glycines* are still unknown. In this study, we assessed the effects of five sublethal concentrations of beta-cypermethrin on various biological traits and demographic parameters of *A. glycines* using a TWISEX-MSChart computer program (Chi 2012). The results of the present study may be helpful for optimizing IPM programs on soybean aphids.

Materials and methods

Study insects

The laboratory colony of *A. glycines* was established from apterous individuals that were collected from a soybean field in Langfang Experimental Station (Hebei province, China) in June 2007. The colony was maintained by continuous supply of insecticide-free soybean seedlings that grew on vermiculite in climate chambers, at 25 ± 2 °C, 60 ± 10 % RH (relative humidity), L: D (light:dark) = 17:7. New soybean seedlings were provided weekly to the colony and aphids were transferred by replacing infested seedlings with insect-free ones (Qu et al. 2015).

Insecticide and acute toxicity assessment

Analytical grade beta-cypermethrin (95%) was purchased from Jiangsu Pesticide Research Institute Co., Ltd (China). Bioassays were conducted using an insect-dipping method according to the Agricultural Industry standard of the People's Republic of China (NY/T 1154.6-2006).

Preliminary experiments were carried out to determine the scope of concentrations to be tested and then six concentrations of beta-cypermethrin (1000, 100, 50, 10, 1 and 0.1 µg/L) were used in the bioassays. Leaf discs (15-mm diameter) were cut from fully expanded insecticide-free soybean leaves, and then placed upside down onto 2% agar into a 12-well tissue-culture plate. Twenty aphids per replicate were dipped into insecticide solution for 10 s, and then introduced into a well that was confined using a filter paper strip (Hangzhou, China) and there were four replicates per tested concentration. Control aphids were dipped into a solution of distilled water containing 0.005% (v/v) Triton X-100 (a non-ionic surfactant) and 0.1% acetone, which is equal to the concentration of acetone in the dipping solution at the highest concentration tested. The mortality was assessed 24 h post-treatment of exposure; aphids that did not move legs when touched with a fine brush, i.e., no reflex movement, were considered dead (Moore et al. 1996). All the experimental arenas were maintained in climate chambers under the same conditions as above. The concentration-mortality (Abbott 1925) regression line, LC₅ and LC₁₅ were calculated to obtain the concentrations for subsequent experiments.

Sublethal concentration assessment

The assessment was carried out using the insect-dipping method, as described above (section *Insecticide and acute toxicity assessment*). Five low concentrations of beta-cypermethrin i.e., 0.625, 1.25, 2.5, 5 (nearly LC₅) and 10 µg/L (nearly LC₁₅) were used during the assays. The mortality rates induced by the five concentrations were compared with those observed in control individuals that were exposed to distilled water containing 0.005% (v/v) Triton X-100 plus 0.001% acetone.

Sublethal effects on biological and demographic parameters

Newly-hatched *A. glycines* nymphs were collected within 24 h after spawning. There were 31, 32, 33, 32, 40, 32 newly-hatched nymphs in control, 0.625, 1.25, 2.5, 5 and 10 µg/L beta-cypermethrin treatment groups, respectively, in the life table experiment. Each individual growing on one insecticide-free soybean seedling was considered as one replicate which was kept in climate chambers, at 25 ± 2 °C, 60 ± 10 % RH, L:D = 17:7. Survival and development of each nymph were recorded every day. On the fifth day after birth, the third instar nymphs in the six groups were exposed to the corresponding concentrations of beta-cypermethrin (0.625, 1.25, 2.5, 5 and 10 µg/L) and control solution, respectively. Survivals and development were recorded daily until the end of the experiment (i.e., when adults died). When

the nymphs became adults, oviposition was also checked until adult death. Every 5 days, the soybean seedlings were replaced by new ones for aphid feeding (Qu et al. 2015).

Data analysis

LC₅, LC₁₅ and LC₅₀ values were calculated using SPSS 20.0 in probit analysis (SPSS Inc, Chicago, USA). Mortality of the apterous adults exposed to beta-cypermethrin was analyzed using SPSS 20.0 in a one-way analysis of variance (ANOVA) followed by a Tukey's HSD test for multiple comparisons to assess the sublethal concentrations of beta-cypermethrin. A two-way ANOVA was processed on the cumulative number of offspring over time exposed to different treatments. Moreover, to compare the differences of cumulative number of offspring among treatments ($P < 0.05$), a Tukey's HSD test was carried out. Raw data from all individuals were analyzed using the TWSEX-MSChart computer program (Chi 2012) according to the age-stage, two-sex life table theory (Chi 1988). Moreover, survival, development, oviposition and population parameters, their mean values and standard errors were estimated by the bootstrap method involved in the TWSEX-MSChart computer program. Significant differences ($P < 0.05$) among different treatments were calculated by Tukey's HSD method included in the TWSEX-MSChart computer program. The curves of survival rates and cumulative number of offspring per female were drawn by Sigmaplot 10.0.

In the two-sex life table theory, the age-stage specific survival rate (S_{xj}) (x = age, j = stage) is the probability that a newly laid egg will survive to age x and stage j . The fecundity value (F_{xj}) is defined as the reproductive rate of an individual in age x and stage j . The life expectancy (E_{xj}) is the time that one individual of age x and stage j is expected to live. The age-specific survival rate (l_x) and the age-specific fecundity (m_x) were also recorded. Among the demographic growth parameters calculated according to Chi and Su (2006) as follows: (i) Net reproductive rate ($R_0 = \sum l_x m_x$), (ii) Intrinsic rate of increase (r_m) can then be estimated with the iterative bisection method from the Euler-Lotka equation, with age indexed from 0 (Goodman 1982) as follows: $\sum_{x=0}^{\infty} e^{-r(x+1)} l_x m_x = 1$. (iii) Finite rate of increase ($\lambda = \exp(r_m)$). (iv) Mean generation time (T) is calculated as $T = \ln R_0 / r_m$.

Results

Acute toxicity of beta-cypermethrin on *A. glycines*

Based on log-probit regression analysis, the regression equation of mortality relative to concentration is $Y = 1.699X + 2.270$ ($\chi^2 = 10.914$, $P = 0.976$). The LC₅₀ of

beta-cypermethrin on third instar *A. glycines* was 46 µg/L (95% confidence interval: 34–59 µg/L). LC₅ and LC₁₅ of beta-cypermethrin on *A. glycines* were 5 µg/L (2–9 µg/L) and 11 µg/L (6–17 µg/L), respectively.

Assessment of sublethal concentrations

The exposure to 0.625, 1.25, 2.5, 5, 10 µg/L beta-cypermethrin and control solution led to 5.76 % (95% confidence interval: 0.83–10.69 %), 7.72% (2.34–13.10 %), 8.97% (6.35–11.59 %), 6.04 % (2.88–9.20 %), 12.1 % (5.51–18.65 %) and 7.74 % (4.09–11.38 %) mortality of aphids, respectively. There was no significant difference in mortality among these exposure concentrations, including control ($F_{5, 24} = 1.940$, $P = 0.125$). Therefore, all these beta-cypermethrin concentrations were considered as sublethal ones according to Desneux et al. (2007).

Effects of sublethal concentrations of beta-cypermethrin on demographic parameters of *A. glycines*

Sublethal beta-cypermethrin concentrations showed little influence on survival rate in the first 8 days after exposure to the insecticide (Fig. 1). However, the survival rate of individuals exposed to 10 µg/L i.e., approximately LC₁₅, declined rapidly on the 14th day after birth, which was 7 days earlier than that of the control. Aphids from the control group survived the longest and the last one survived until the 28th day, while the last aphid of 0.625, 1.25, 2.5, 5 and 10 µg/L beta-cypermethrin treatment groups died on the 27th, 23rd, 27th, 26th and 21st day, respectively.

Effects of sublethal concentrations of beta-cypermethrin on the development duration in young instar, adult longevity and oviposition period of *A. glycines* are reported in

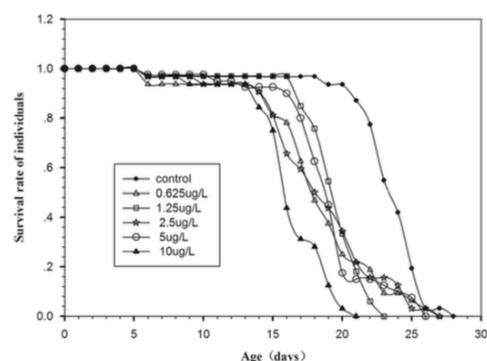


Fig. 1 The survival rate of *A. glycines* exposed to five sublethal concentration of beta-cypermethrin (and water as control)

Table 1. The 10 µg/L beta-cypermethrin significantly prolonged the development time of aphids in the 4th instar. However, the sublethal concentrations of beta-cypermethrin significantly shortened the adult lifespan and oviposition period of *A. glycines*. In addition, the 10 µg/L beta-cypermethrin had a strongest negative effect on both lifespan and oviposition period of *A. glycines*, which were significantly shorter than those of other sublethal concentrations.

The result of two-way ANOVA showed that concentration has a significant effect ($F_5 = 17.87$, $P < 0.001$) on the cumulative number of offspring over time. The cumulative numbers of offspring per adult in 0.625, 1.25, 2.5, 5 and 10 µg/L beta-cypermethrin treatment groups and control were (i) 60.27 (95% confidence interval: 57.57–62.97); (ii) 58.28 (56.17–60.39); (iii) 63.16 (59.27–67.06); (iv) 75.41 (72.46–78.36); (v) 52.52 (49.25–55.78) and (vi) 62.97 (60.65–65.29) respectively. The cumulative number of offspring in 5 µg/L beta-cypermethrin treatment group significantly increased but it significantly decreased in 10 µg/L treatment group compared to control and other treatments (Fig. 2).

Effects of sublethal concentrations of beta-cypermethrin on the demographic parameters of *A. glycines* are reported in Table 1. Net reproduction rate (R_0) of the aphids exposed to 5 µg/L beta-cypermethrin (73.521 ± 2.348) significantly increased while R_0 in 10 µg/L beta-cypermethrin treatment group (50.863 ± 2.214) significantly decreased compared to the control (60.975 ± 2.262) and other sublethal concentration treatments (0.625, 1.25 and 2.5 µg/L), whose R_0 were 56.459 ± 2.846 ; 56.535 ± 1.985 and 61.184 ± 2.709 , respectively. In addition, intrinsic rate of increase (r_m) of *A. glycines* exposed to 5 µg/L beta-cypermethrin (0.429 ± 0.003) was significantly higher than that of the control (0.409 ± 0.006) and other sublethal concentration treatments. Similar results were achieved on finite rate of increase (λ), i.e., the λ of *A. glycines* treated with 5 µg/L beta-cypermethrin (1.536 ± 0.006) was significantly higher than that of the control (1.505 ± 0.008) and other sublethal concentrations. Mean generation time (T) of aphids exposed to 1.25 µg/L (9.742 ± 0.088) and 10 µg/L (9.665 ± 0.095) was significantly shorter than that of the control (10.05 ± 0.099), while the other sublethal concentrations showed no significant differences in T values.

Discussion

In this study, sublethal and stimulatory effects of beta-cypermethrin at sublethal concentrations on *A. glycines* were evaluated. We demonstrated that these concentrations decreased the adult lifespan and reproductive period of *A. glycines*. These results concur with the recent research

Table 1 Effects of sublethal concentrations of beta-cypermethrin on development duration and demographic parameters of *A. glycines*

| Concentration (µg/L) | Demographic parameters | | | | | | | | | |
|----------------------|------------------------|---------------|---------------|---------------|-----------------|--------------------|---------------------------------|--------------------------------------|---------------------------------------|--------------------------|
| | Stages (days) | | | | | | | | | |
| | First-instar | Second-instar | Third-instar | Forth-instar | Adult longevity | Oviposition period | Net reproductive rate (R_0) | Intrinsic rate of increase (r_m) | Finite rate of increase (λ) | Mean generation time (T) |
| 0.625 | 2.09 ± 0.05 a | 1.91 ± 0.05 a | 1.12 ± 0.06 a | 1.00 ± 0.00 b | 13.10 ± 0.59 b | 9.30 ± 0.28 cd | 56.459 ± 2.846 b | 0.407 ± 0.006 b | 1.503 ± 0.009 b | 9.899 ± 0.085 ab |
| 1.25 | 2.12 ± 0.06 a | 1.91 ± 0.05 a | 1.12 ± 0.06 a | 1.00 ± 0.00 b | 13.65 ± 0.30 b | 9.72 ± 0.23 c | 56.535 ± 1.985 b | 0.414 ± 0.005 b | 1.513 ± 0.007 b | 9.742 ± 0.088 bc |
| 2.5 | 2.13 ± 0.06 a | 1.88 ± 0.06 a | 1.13 ± 0.06 a | 1.00 ± 0.00 b | 12.87 ± 0.68 b | 9.51 ± 0.36 c | 61.184 ± 2.709 b | 0.410 ± 0.004 b | 1.507 ± 0.008 b | 10.03 ± 0.110 a |
| 5 | 2.08 ± 0.04 a | 1.87 ± 0.05 a | 1.10 ± 0.05 a | 1.00 ± 0.00 b | 13.37 ± 0.49 b | 10.84 ± 0.27 b | 73.521 ± 2.348 a | 0.429 ± 0.003 a | 1.536 ± 0.006 a | 10.02 ± 0.077 a |
| 10 | 2.09 ± 0.05 a | 1.91 ± 0.05 a | 1.06 ± 0.04 a | 1.03 ± 0.03 a | 10.58 ± 0.42 c | 8.74 ± 0.34 d | 50.863 ± 2.214 c | 0.406 ± 0.005 b | 1.502 ± 0.007 b | 9.665 ± 0.095 c |
| control | 2.10 ± 0.05 a | 1.90 ± 0.05 a | 1.13 ± 0.06 a | 1.00 ± 0.00 b | 17.77 ± 0.37 a | 13.31 ± 0.33 a | 60.975 ± 2.262 b | 0.409 ± 0.006 b | 1.505 ± 0.008 b | 10.05 ± 0.099 a |

Data (mean ± SE) were analyzed using Twosex-MSCchart and followed by Tukey test. Within a column, different letters indicate significant differences ($P < 0.05$)

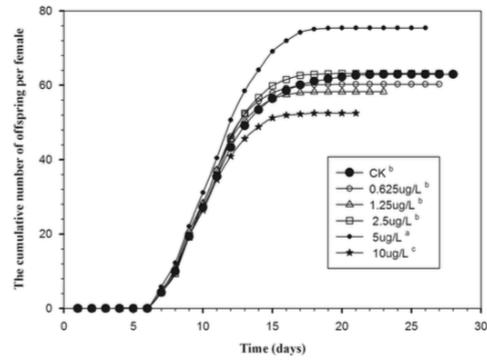


Fig. 2 The cumulative number of offspring per *A. glycines* adult exposed to sublethal concentrations of beta-cypermethrin. The cumulative number of offspring per adult was analyzed using two-way ANOVA over the time among different treatments and followed by Turkey HSD test to compare the difference among different treatments. The curves of cumulative number of offspring with different letters indicated significant difference at $P < 0.05$ level

showing that low concentrations of beta-cypermethrin reduced oviposition period and adult male longevity in *P. xylostella* (Song et al. 2013). Similar findings have been reported for other pyrethroid insecticides e.g. *P. xylostella* adult longevity significantly decreased by 36% when larval stages were treated with LC₁ of permethrin and fenvalerate (Kumar and Chapman 1984). Sublethal concentration of Cyhalothrin (LC₂₀) shortened the adult lifespan of *Adelphocoris suturalis* and prolonged their hatching periods (Li et al. 2008). In addition, both repellent and antifeedant activities were detected on larvae of *P. xylostella* exposed to sublethal concentrations of pyrethroid. Therefore, pyrethroids probably reduced insect fitness by lowering its feeding activity, thus negatively affecting uptake of nutrients.

In our study, a significantly stimulatory effect on fecundity was obtained when the aphids were exposed to 5 µg/L beta-cypermethrin (LC₅), the exposed adults producing more offspring than in the control group; therefore pesticide-induced hormesis may occur in *A. glycines* when exposed to beta-cypermethrin in soybean fields. Similar hormesis has been reported in *P. xylostella* which laid more eggs when exposed to sublethal doses of the pyrethroid fenvalerate (Sota et al. 1998; Fujiwara et al. 2002). Such stimulatory effect on fecundity may also occur in case of natural enemies; the cumulative numbers of offspring increased by 49.64% (compared to the control) after ladybird beetles were exposed to a sublethal concentration of beta-cypermethrin (LC₅) (Xiao et al. 2016). The possible physiological mechanisms of stimulatory effects on

reproduction were closely related to the insect hormone and change in the transcription level of vitellogenin. The levels of juvenile hormone III in adult aphids which fed on leaves treated with sublethal concentrations of imidacloprid showed a similar trend to that of fecundity (Yu et al. 2010).

Life table is one of the most useful tools in the research of pest population dynamics (Carey 1993). In our study, stimulating effects on population growth are more obvious when the sublethal concentration of beta-cypermethrin increases. The largest stimulatory effect on fecundity was achieved when aphids were exposed to the sublethal concentration of 5 µg/L (LC₅). Net reproductive rate (R_0), intrinsic rate of increase (r_m) and finite rate of increase (λ) of the *A. glycines* population all significantly increased. However, the population growth was inhibited when the sublethal concentration continued to increase to 10 µg/L (LC₁₅). Our results are consistent with previous studies. The soybean population treated with imidacloprid at a concentration of 0.05 mg/L showed enhanced net reproductive rate (R_0). The reversed effect on R_0 was achieved when soybean aphids were exposed to 0.2 mg/L imidacloprid (Qu et al. 2015). Moreover, it has also been found that beta-cypermethrin at a sublethal concentration (LC₅) increased R_0 and r_m of *Harmonia axyridis* population in F1 generation (Xiao et al. 2015, 2016). However, various studies reported contrasted results on different pests and/or different pesticides. The R_0 , r_m and λ of the *P. xylostella* population treated with LC₂₅ of chlorantraniliprole were remarkably lower than those of the control (Guo et al. 2013). Different effects on demographic parameters may be linked to the action modes of pesticides, exposure doses of pesticides, pest species, and pest feeding ways used in researches.

Hormesis in insects exposed to sublethal concentrations of insecticides has been documented for several taxa and compounds (Tan et al. 2012; Qu et al. 2015; Xiao et al. 2015), especially for pyrethroids (Cutler 2013). In the present study, we have assessed whether hormesis effects, especially on fecundity traits, may occur in *A. glycines* during and/or after exposure to sublethal concentrations of beta-cypermethrin. Our findings indicated that a high sublethal concentration of beta-cypermethrin (5 µg/L) increased fecundity by 19 % (12–28 %) compared with control. By contrast, beta-cypermethrin at the highest sublethal concentration (10 µg/L) decreased the fecundity by 17 % (8–25%). Our results concur with a previous study on citrus thrips; high malathion residue concentrations initially inhibited fecundity during exposure while this reproductive trait was increased post-exposure (Morse and Zareh 1991). The mechanism(s) underneath insecticide-induced hormesis effect in insects may be linked to conservation laws in mass and energy. The insects have to allocate energy/mass to detoxify insecticides at

sublethal doses. Some life-history traits such as development and reproduction are changed as a result of responses to the stimulation (Jager et al. 2013). Pyrethroid (Cordeiro et al. 2013) and neonicotinoid insecticides (Yu et al. 2010) have been reported to cause a hormesis on pests, especially on the reproduction trait. Exposure to sublethal concentrations of imidacloprid could stimulate reproduction on *Myzus persicae* (Cutler et al. 2009; Rix et al. 2016) and *A. glycines* (Qu et al. 2015). In addition, the applications of synthetic pyrethroids induce resurgence of *Aphis gossypii* on cottons (Nandihalli et al. 1992). Recently, deltamethrin-induced hormesis has been linked to red mite outbreaks (Cordeiro et al. 2013). Still, resurgence of a given pest can be also attributed to various biotic and abiotic factors and not necessarily induced by hormesis. For example, non-target harmful effects of pesticides on arthropod natural enemies could be largely responsible (Desneux et al. 2007). Frequent insecticide sprayings could also induce pest population resurgence by increasing the pest resistance and disrupting the biocontrol services provided by arthropod natural enemies in agroecosystems (Lu et al. 2012).

Overall, our results on *A. glycines*, together with the evidence of other insecticides on pests (Fujiwara et al. 2002; Cutler et al. 2009), suggest that insecticide-induced hormesis should be recognized as one of the mechanisms explaining arthropod pest resurgence (Cutler 2013; Guedes et al. 2016). The resistant genotypes can survive after insecticide spray. Thereafter, the stimulatory effect on development and growth of resistant insects, i.e., hormesis, may favor the population growth of these individuals. Therefore, hormesis is an unfavorable phenomenon to insecticide resistance management and may shorten the lifespan of a given insecticidal compound, especially when it is used frequently. Key changes in biochemical endpoints have recently been analyzed during pesticide-induced stimulation (Cutler 2013; Guedes et al. 2016). However, gene expression during the sublethal effects and hormesis has not been fully studied in insect-insecticide models. Thus, further research on the molecular mechanisms of sublethal effects and insecticide-induced hormesis is required.

Acknowledgements We thank National Natural Science Foundation of China (Grant number: 31272077) for financially supporting the research presented in this article. We are grateful to Professor Hsin Chi (Laboratory of Theoretical and Applied Ecology, Department of Entomology, National Chung Hsing University, Taiwan) for providing the Age-Stage Two-Sex Life-Table program.

Funding This study was funded by the National Natural Science Foundation of China (Grant number: 31272077).

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Acetamiprid-induced hormetic effects and vitellogenin gene (*Vg*) expression in the melon aphid, *Aphis gossypii*

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With 4 figures and 4 tables

Abstract: Stimulatory effects (hormesis) of acetamiprid on demographical traits and vitellogenin gene expression of melon aphid, *Aphis gossypii* Glover, were examined. The longevity and fecundity of the parental aphids (F_0) were both reduced by a low lethal concentration of acetamiprid (LC_{15}), while no effect was observed when exposed to a sublethal concentrations (LC_5). However, acetamiprid-induced hormetic effects were observed in the F_1 generation. The pre-adult developmental period and fertility of progeny aphids were increased by both concentrations (LC_5 and LC_{15}). Subsequently, key demographic parameters such as intrinsic rate of increase (r) and finite rate of increase (λ) were increased by the LC_5 , and the mean generation time (T) and net reproductive rate (R_0) were increased by both concentrations of acetamiprid. Furthermore, both acetamiprid concentrations induced the expression level of *Vg* gene in the progeny generation. The higher mRNA transcript level of *Vg* might be translated into an increased reproduction of F_1 generation in *A. gossypii*. Based on the results, acetamiprid at low or sublethal concentrations may induce stimulatory effects on the subsequent generations of *A. gossypii* along with the increased transcription of *Vg* gene. These findings may help understanding the hormetic effects of acetamiprid in insects and could help optimizing the use of this insecticide against *A. gossypii*.

Keywords: Biological traits; hormesis; neonicotinoid; fecundity; longevity

1 Introduction

The melon Aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae), is a cosmopolitan and deleterious pest of numerous vegetable plants throughout the world (Hullé et al. 2019). The melon aphid can cause damage through direct feeding and indirectly by transmitting several plant pathogenic viruses (Pitrat & Lecoq 1980; Campolo et al. 2014). Various tactics have been implemented (e.g., see Lu et al. 2012; Han et al. 2014; Ali et al. 2016), but chemical applications have a pivotal role in the current management of this pest (Chen et al. 2016; Yuan et al. 2017). However, the indiscriminate application of chemical insecticides resulted in the development of resistance in *A. gossypii* (Shrestha & Parajulee 2013; Koo et al. 2014; Tang et al. 2017; Tieu et al. 2017).

Acetamiprid belongs to the neonicotinoid group of insecticides developed by Nippon Soda Co., Ltd, interfering with the nicotinic acetylcholine receptor (nAChR) of the insect nervous system (Tomizawa & Casida 2005). This insecticide

has been used extensively for the control of many sucking insect pests (Khattak et al. 2004; Raghuraman et al. 2008; Chen et al. 2018), although it starts being banned in few places, e.g. in France (Jactel et al. 2019). Pesticides commonly lead to direct mortality in insect pests. However, misapplication, suboptimal spray or residual levels present after initial application of pesticides under field conditions (Desneux et al. 2005) may lead the insects to be exposed to low or sublethal concentrations of these compounds (Desneux et al. 2007). These sublethal exposures may impact negatively physiology and/or behavior traits in surviving arthropods, which ultimately could affect their population growth (Desneux et al. 2006; 2007; Guo et al. 2013; Biondi et al. 2015). For example, reduced insect longevity and fecundity have been observed in various arthropods (Wang et al. 2017; Cui et al. 2018; Luo et al. 2018; Parreira et al. 2018; Passos et al. 2018; Zhang et al. 2018). Such effects of pesticides could steadily decrease the ecosystem services provided by key beneficial arthropods (Desneux et al. 2007; Lu et al. 2012; Decourtye

et al. 2013; Mohammed et al. 2018). However, various studies have reported increased fecundity in several insect pests after exposure to sublethal concentrations of insecticides (Tan et al. 2012). These stimulatory effects induced by pesticides are known as "pesticide-induced hormesis" (Guedes et al. 2016). Hormesis (stimulatory effects) is a phenomenon characterized by low-dose stimulation and high-dose inhibition (Calabrese & Blain 2005; Calabrese 2005). Numerous studies reported such effects, e.g., sublethal concentrations of nitenpyram, pirimicarb, and flonicamid can induce stimulation of reproduction in *A. gossypii* (Koo et al. 2015; Wang et al. 2017). Hormetic effects have also been documented for sublethal concentrations of flupyradifurone, imidacloprid and precoene in *Myzus persicae* Sulzer (Hemiptera: Aphididae) (Yu et al. 2010; Ayyanath et al. 2015; Tang et al. 2019).

So far, possible hormetic effects have not been studied for acetamiprid insecticides in melon aphids. Therefore, an age-stage, two-sex life table has been used to investigate the hormetic effects of acetamiprid (LC₅ and LC₁₅) on the demographical parameters of *A. gossypii*. Furthermore, the relative transcriptional level of *Vg* was determined, which plays an integral role in insect reproduction (Yan et al. 2016; Zhen et al. 2018). These results would be useful to understand the putative hormetic effects of acetamiprid on the biological traits of *A. gossypii*.

2 Materials and methods

2.1 Insects and insecticides

The melon aphid colony used in this study was collected from melon plants. Aphids were reared and maintained on insecticide-free cucumber plants. The temperature was maintained at 25 ± 1 °C and 70% ± 10% relative humidity (RH), while the light/dark photoperiod was 16:8 h. All experiments were performed in the abovementioned controlled environmental conditions. Technical grade acetamiprid (97.5%) was obtained from Jiangsu Anpon Electrochemical Co., Ltd. China.

2.2 Insecticide exposure

A leaf-dip bioassay technique was used to investigate the toxicity of acetamiprid on *A. gossypii* (Moores et al. 1996). Primarily, the mother solution of acetamiprid was prepared using acetone, then distilled water containing 0.05% (v/v) Triton X-100 was used to further dilute the acetamiprid solution. Preliminary bioassay experiments were carried out to estimate the concentrations to be used in subsequent experiments. Five different concentrations of acetamiprid (2, 1, 0.5, 0.25, and 0.125) were used in the bioassay experiments. Distilled water containing 0.05% (v/v) Triton X-100 was used as a control. Each treatment had three replications, and at least 20 aphids per replicate were used in the bioassay.

Fresh cucumber leaf discs were dipped for 15 s in the required solutions of acetamiprid and control. After air drying, the exposed discs were placed on 2% agar gel (2 ml) in a 12-well tissue culture plate (Wang et al. 2017; Tang et al. 2019). Adult melon aphids (≤ 24 h old) were inoculated onto treated leaf discs. Parafilm (Chicago, USA) was used to prevent insect escape. The plates were placed under the laboratory conditions (25 ± 1 °C, 75% RH, 16:8/L:D). Insect mortality was recorded after 72 h exposure. Individual aphids that did not show any movement when pushed gently were counted as dead (Moores et al. 1996). Every bioassay experiment was repeated three times. The dose–response for acetamiprid was calculated by using PoloPlus 2.00.

2.3 Sublethal and transgenerational effects of acetamiprid on melon aphid F₀ and F₁ generations

This experiment aims to investigate the sublethal effects of acetamiprid on the demographical parameters of directly exposed F₀ melon aphids and their descendants (F₁). In this context, we choose low lethal (LC₁₅) and sublethal (LC₅) concentrations of acetamiprid (obtained from the above experiments) likely to occur in the field after the degradation of insecticides (Desneux et al. 2005). The abovementioned experimental procedure was used with minor changes (Ou et al. 2015). Fresh leaf discs of cucumber plants were dipped in the LC₅ and LC₁₅ solutions of acetamiprid for 15 s. Distilled water containing 0.05% (v/v) Triton X-100 was used as a control treatment. The treated leaf discs were dried at room temperature and then placed on 2% agar gel (2 ml) in a 12-well tissue culture plate. Adult aphids were transferred to the leaf discs treated with acetamiprid (LC₅ and LC₁₅) and control. Sixty surviving individuals were collected randomly after 72 h exposure and transferred to insecticide-free leaf discs (Yousaf et al. 2018; Tang et al. 2019). Fresh leaf discs were replaced every third day. All the experimental arenas from the treatment groups of LC₅, LC₁₅, and the control were placed under the laboratory conditions mentioned above. During this experiment, the Parental aphids' (F₀) longevity and fecundity were recorded on a daily basis until the death of the adult. To investigate the transgenerational effects of acetamiprid on F₁ melon aphids, sixty neonate nymphs were randomly selected from the preceding generation (LC₅, LC₁₅, and control) and placed individually on fresh cucumber leaf discs without any exposure to insecticides. Leaf discs were replaced every third day throughout the experiment. When aphids became adults and started reproducing, the number of neonate nymphs per aphid was noted daily until the adults died.

2.4 Quantitative Real-Time PCR

Total RNA was isolated from adult *A. gossypii* using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. The cDNA was synthesized from total

RNA (1 ug) using the PrimeScript® RT Reagent Kit with the gDNA Eraser (Takara, Dalian, China). Quantitative reverse transcription PCR (RT-qPCR) reactions were performed using the SYBR® Premix Ex Taq™ (Tli RNaseH Plus) (Takara, Dalian, China) on the ABI 7500 Real-Time PCR system (Applied Biosystems, USA). All the primers used in this study are listed in Table 3. PRIMER 3.0 (<http://bioinfo.ut.ee/primer3-0.4.0/>) was used to design all qPCR primers. Elongation factor 1 alpha (*EF1α*), beta-actin (*β-ACT*) was used as an internal reference gene (Ma et al. 2016). RT-qPCR was performed in a 20 μL reaction consisting of 1 μL of the cDNA template, 0.4 μL of each primer, 0.4 μL of ROX Reference Dye II, 10 μL of SYBR®Premix Ex Taq and 7.8 μL of RNase-free water. The cyclic thermal procedure was performed under the following conditions (Ma et al. 2019a; Ma et al. 2019b): 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s, and then a dissociation stage was performed at 95 °C for 15 s, 60 °C for 1 min, 95 °C for 30 s, and 60 °C for 15 s. The RT-qPCR analysis included three independent biological replicates with three technical replications. The relative gene transcriptional level was calculated using the 2^{-ΔΔCt} method (Livak & Schmittgen 2001).

3 Data Analysis

The LC₅, LC₁₅, LC₅₀ and LC₉₀ of acetamiprid were calculated using a log-probit model (Desneux et al. 2004a), widely used in previous studies (Desneux et al. 2004b; Qu et al. 2015; Tang et al. 2019). The raw data of each *A. gossypii* (adult longevity and fecundity) were analyzed according to an age-stage, two-sex life table (Chi & Liu 1985; Chi 1988) using the TWISEX-MSChart computer program (Chi 2018). Life table parameters (s_{xj} , v_{xj} , l_x , m_x and $l_x m_x$) and demographical traits (r , λ , R_0 , T and GRR) were calculated

according to (Chi & Liu 1985). For precise estimation, the Paired bootstrap method was applied to calculate the mean and standard error of *A. gossypii* demographic parameters in TWISEX-MSChart (Chi 2018). 100,000 replications were used in the bootstrapping procedures (Akca et al. 2015; Akköprü et al. 2015). For RT-qPCR, the statistical differences were determined by one-way ANOVA with a subsequent Tukey post hoc test ($P < 0.05$) (IBM, SPSS Statistics). Sigma Plot 12.0 was used to generate the graphs.

4 Results

4.1 Toxicity of acetamiprid against melon aphids

The LC₉₀, LC₅₀, LC₁₅, and LC₅ values of acetamiprid against melon aphids are reported in Table 1. Acetamiprid was toxic to the tested strain of melon aphids after 72 hours of exposure, with LC₉₀ and LC₅₀ values of 1.71 and 0.378 mg L⁻¹, respectively. The estimated values of LC₁₅ and LC₅ were 0.111 mg L⁻¹ and 0.054 mg L⁻¹ after 72 hours exposure, respectively (Table 1). The LC₅ and LC₁₅ values of acetamiprid were selected to study hormesis effects on demographic parameters and the expression of detoxification genes in melon aphids.

4.2 Impact of low or sublethal concentration of acetamiprid on parental (F₀) and F₁ generations of *A. gossypii*

The exposure of melon aphid (*A. gossypii*) to the low lethal (LC₁₅) concentration of acetamiprid significantly reduced the longevity ($F_{2, 25} = 10.16$; $P < 0.001$) and fecundity ($F_{2, 25} = 18.43$; $P < 0.001$) of the exposed individuals (F₀ generation), while no effects were observed for the sublethal (LC₅) concentration (Table 2). The longevity of the F₁ generation was not significantly affected by either of the acetamiprid

Table 1. Toxicity of acetamiprid against adult *Aphis gossypii*, determined by leaf-dip bioassay.

| Treatments | Slope ± SE ^a | LC ₅ mg L ⁻¹ (95% CL) ^b | LC ₁₅ mg L ⁻¹ (95% CL) ^b | LC ₅₀ mg L ⁻¹ (95% CL) ^b | LC ₉₀ mg L ⁻¹ (95% CL) ^b | χ ² (df) ^c | P |
|-------------|-------------------------|---|--|--|--|----------------------------------|-------|
| Acetamiprid | 1.954 ± 0.245 | 0.05 (0.02–0.09) | 0.11 (0.05–0.16) | 0.38 (0.27–0.48) | 1.71 (1.17–3.25) | 16.505 (13) | 0.223 |

^a Standard error.

^b 95% confidence intervals.

^c Chi-square value (χ²) and degrees of freedom (df) as calculated by PoloPlus 2.0.

Table 2. Developmental time, adult longevity, and fecundity of the F₁ generation of *Aphis gossypii* when parental aphids (F₀) were treated with LC₅ and LC₁₅ of acetamiprid, compared to the untreated control.

| T | 1 st instar | 2 nd instar | 3 rd instar | 4 th instar | Pre-adult | Adult Longevity F ₁ | Adult Fecundity F ₁ | Adult Longevity F ₀ | Adult Fecundity F ₀ |
|------------------|------------------------|------------------------|------------------------|------------------------|------------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|
| Control | 1.80±0.08c | 1.52±0.09b | 1.17±0.07b | 1.40±0.07c | 5.88±0.10c | 20.07±0.66a | 36.05±0.77b | 16.05±0.68a | 27.71±0.75a |
| LC ₅ | 1.90±0.09ab | 1.32±0.08b | 1.38±0.10b | 1.80±0.07a | 6.40±0.13b | 21.23±0.88a | 40.20±0.84a | 15.01±0.81a | 25.80±0.71a |
| LC ₁₅ | 2.20±0.09a | 1.92±0.12a | 1.90±0.13a | 1.60±0.08ab | 7.62±0.13a | 21.82±0.67a | 42.40±0.88a | 11.60±0.68b | 21.10±0.89b |

Different letters within the same column represent significant differences at $P < 0.05$ level (one-way ANOVA followed by Tukey's HSD test).

Table 3. Transgenerational effects of acetamiprid on population parameters of the F_1 generation of *Aphis gossypii* whose parents (F_0 generation) were treated with LC_5 and LC_{15} concentrations, compared to untreated control.

| Parameters ^a | Control (Mean±SE ^b) | LC_5 (Mean±SE ^b) | P value | Control (Mean±SE ^b) | LC_{15} (Mean±SE ^b) | P |
|-------------------------|---------------------------------|--------------------------------|---------|---------------------------------|-----------------------------------|--------|
| r | 0.318 ± 6.523b | 0.335 ± 5.577a | 0.048 | 0.318 ± 6.523a | 0.295 ± 4.904b | 0.006 |
| λ | 1.374 ± 8.969b | 1.398 ± 7.798a | 0.048 | 1.374 ± 8.969a | 1.344 ± 6.594b | 0.006 |
| R_0 | 36.052 ± 0.763b | 40.193 ± 0.834a | <0.001 | 36.052 ± 0.763b | 42.400 ± 0.879a | <0.001 |
| T | 11.269 ± 0.231a | 11.025 ± 0.184a | 0.406 | 11.269 ± 0.231b | 12.674 ± 0.208a | <0.001 |
| GRR | 44.112 ± 1.162a | 44.798 ± 0.952a | 0.647 | 44.112 ± 1.162a | 46.300 ± 0.983a | 0.186 |

^a r : intrinsic rate of increase (d^{-1}), λ : finite rate of increase (d^{-1}), R_0 : net reproductive rate (offspring/individual), T : mean generation time, GRR : gross reproduction rate.

^b Standard errors (SE) were estimated by using the bootstrap technique with 100,000 re-samplings.

Different letters within the same row show significant differences between control and acetamiprid concentration groups (at the $P < 0.05$ level, paired bootstrap test using TWSEX MS chart program).

Table 4. Specific primers used for qRT-PCR.

| Primer name | Primer sequences |
|----------------------------------|------------------------|
| Vg-F | GGCGATAGTTCGATGAAAA |
| Vg-R | TTTTTGGCATCCACAATCA |
| <i>EF1α</i> -F | GAAGCCTGGTATGGTTGTCTGT |
| <i>EF1α</i> -R | GGGTGGGTGTCTTCTTGTG |
| β -Actin-F | GGGAGTCATGGTTGGTATGG |
| β -Actin-R | TCCATATCGTCCAGTTGGT |

concentrations, as compared to the control group ($F_{2, 178} = 1.410$; $P = 0.246$) (Table 2). However, the fecundity of F_1 individuals was significantly increased at both concentrations ($F_{2, 178} = 14.97$; $P < 0.001$) (Table 2). The developmental durations of the first ($F_{2, 178} = 5.19$; $P = 0.006$), second ($F_{2, 178} = 9.13$; $P < 0.001$) and third instars ($F_{2, 178} = 13.23$; $P < 0.001$) were significantly increased compared to the control group, after exposure of the parental generation (F_0) to LC_{15} of acetamiprid (Table 2). The developmental duration of the fourth instar nymph was also prolonged by both of the acetamiprid concentrations ($F_{2, 178} = 6.21$; $P = 0.002$) (Table 2). Furthermore, the pre-adult period was significantly increased ($F_{2, 178} = 51.04$; $P < 0.001$) after treatment by both concentrations of acetamiprid, in comparison with control (Table 2).

4.3 Transgenerational impact of acetamiprid on the F_1 generation of *A. gossypii*

Transgenerational effects of LC_5 and LC_{15} of acetamiprid on the demographic parameters of the F_1 generation of *A. gossypii* were estimated with a paired bootstrap test using the TWSEX MS chart program (Chi 2018) and are reported in Table 3. The estimated intrinsic rate of increase (r), finite rate of increase (λ) and net reproduction rate (R_0) increased significantly in F_1 individuals when the parental generation (F_0) had been previously exposed to LC_5 of acetamiprid (Table 3). However, no statistical differences were observed in the mean generation time (T) and gross reproductive rate

(GRR) at LC_5 of acetamiprid compared to the control group (Table 3). By contrast, the intrinsic rate of increase (r) and finite rate of increase (λ) decreased, and the net reproduction rate (R_0) and mean generation time (T) increased significantly in the F_1 generation at LC_{15} of acetamiprid, while there was no significant difference in the gross reproductive rate (GRR), as compared to the control group (Table 3).

The age-stage specific survival rate (s_{xj}) (Fig. 1) shows the probability that a newly-born nymph will survive to age x and stage j . Because of the variable developmental rates of melon aphids, overlaps in immature stages were obviously observed in control (Fig. 1A), LC_5 (Fig. 1B) and LC_{15} (Fig. 1C) of acetamiprid. However, the adult survival rate is different among the control, LC_5 , and LC_{15} groups.

The age-specific survival rate (l_x), age-specific fecundity (m_x) and age-specific maternity ($l_x m_x$) were also measured in the LC_{15} and LC_5 -treated melon aphids, as compared to the control group (Fig. 2). The age-specific survival rate (l_x) showed a simplified form of the survival history and the probability that a newly-born nymph will survive to age x (Fig. 2A). The l_x started to decline on day 14 for the control group, whereas in both treatments LC_5 and LC_{15} of acetamiprid, the population started to decline on day 15 (Fig. 2A). However, a higher l_x of F_1 melon aphids was observed in the LC_{15} -treated group from age 16 to 40 day. While in the case of LC_5 , a higher l_x was observed from age 21 to 40 day (Fig. 2A). The age-specific fecundity (m_x) of the LC_{15} treated individuals were higher from day 8 to day 21. Meanwhile, in LC_5 a higher m_x value was observed from day 7 to day 15 (Fig. 2B). A similar trend of age-specific maternity ($l_x m_x$) was observed in both treatments of acetamiprid and the control group (Fig. 2C).

The age-stage reproductive values (v_{xj}) of LC_5 (Fig. 3B) and LC_{15} (Fig. 3C) of acetamiprid-treated adult melon aphids were different from the adults of the control group (Fig. 3A). The maximum v_{xj} values for LC_5 and LC_{15} were 12.4 at the age of the 4th day and 14.1 at the age of the 5th day, respectively, which were higher than the maximum v_{xj} value of 8.6 at the age of the 5th day in the control group (Fig. 3). In

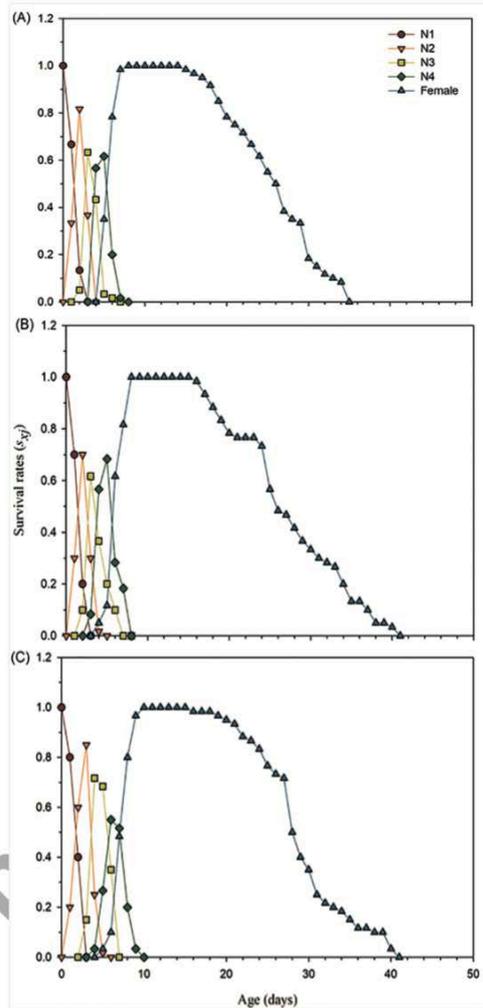


Fig. 1. Age-stage specific survival rate (s_{xj}) for F_1 generation *Aphis gossypii* descending from parents (F_0) under control condition (A), treated with LC_5 (B), and LC_{15} (C) of acetamiprid.

addition, it was found that in LC_5 and LC_{15} of acetamiprid, the reproductive durations of F_1 melon aphids were 15 and 17 days with the v_{xj} value at more than 6, respectively, while in the control group, the reproductive duration was 14 days with the v_{xj} value at more than 6 (Fig. 3).

4.4 Expression of the *Vg* gene at low or sublethal concentrations of acetamiprid

The hormetic effects of low or sublethal concentrations of acetamiprid (LC_5 and LC_{15}) on the *Vg* gene of the parental (F_0) and the progeny generation (F_1) of *A. gossypii* were

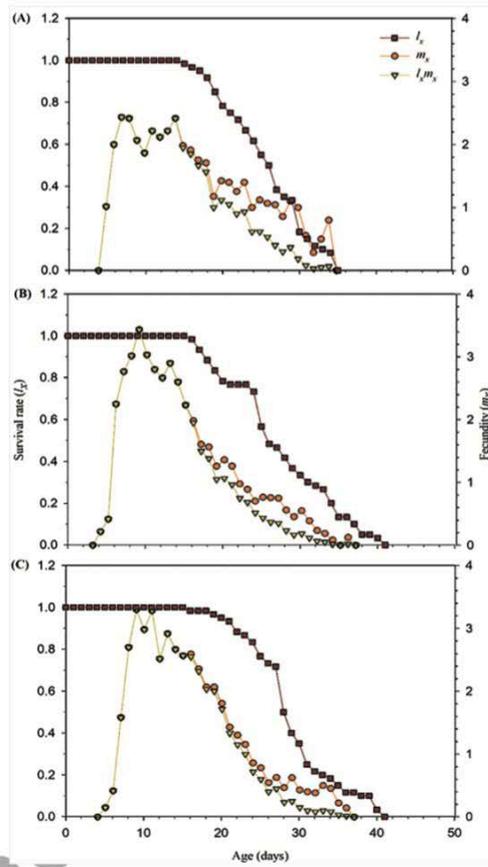


Fig. 2. Population age-specific survival rate (l_x), age-specific fecundity (m_x) and the age-specific maternity ($l_x m_x$) for F_1 generation *Aphis gossypii* descending from F_0 individuals exposed to the LC_5 and LC_{15} of acetamiprid.

evaluated following 72 h exposure (Fig. 4). Results showed that the transcriptional level of the *Vg* gene was not affected in the parental aphids as compared to the control individuals (Fig. 4). However, a significant increase in the abundance of the *Vg* mRNA transcript was observed in the progeny generation (F_1) melon aphids. The transcriptional level of the *Vg* gene was increased up to 1.91- and 2.19-fold after exposure to LC_5 and LC_{15} of acetamiprid for 72 h, respectively, compared to the control (Fig. 4).

5 Discussion

Insecticide-induced hormesis is a biphasic phenomenon, characterized by low-dose stimulation and high-dose inhibition, which has been chosen by many organisms for their survival and reproduction in stressful environmental conditions (Rix et al. 2016; Sial et al. 2018). In this study, we investigated the sublethal and stimulatory effects of acetamiprid, at low or sublethal concentrations, on melon aphids. We

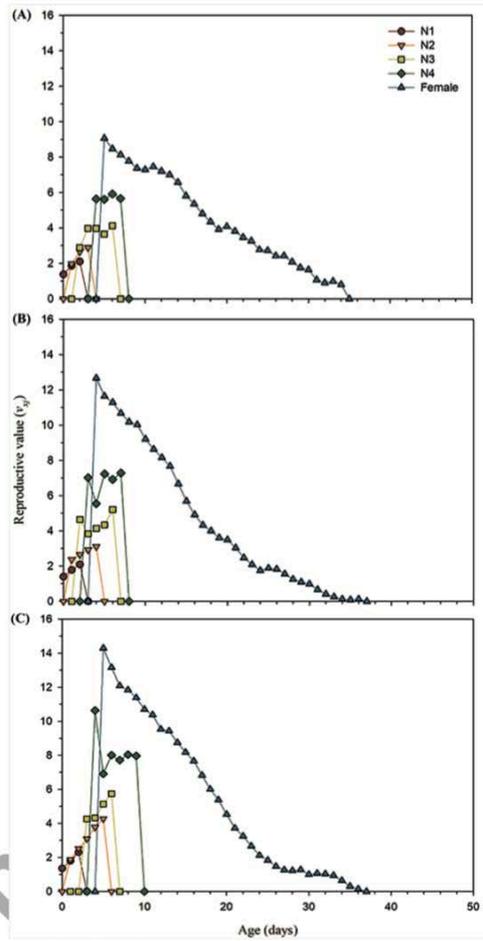


Fig. 3. Age-stage reproductive value (v_{xj}) for F_1 generation *Aphis gossypii* produced from parents (F_0) under control conditions (A), treated with LC_5 (B) and LC_{15} of acetamiprid.

demonstrated that LC_5 and LC_{15} of acetamiprid significantly increased reproduction in the F_1 generation descending from the exposed parental generation (F_0). All these results support the hypothesis that hormesis was involved in *A. gossypii*, likely a manifestation of adaptive mechanisms followed by low or sublethal exposure to acetamiprid insecticide (Sial et al. 2018).

In this study, adverse effects of acetamiprid on the F_0 generation of *A. gossypii* were observed when exposed to the low lethal (LC_{15}) and sublethal (LC_5) concentrations for 72 hours. Acetamiprid applied at low or sublethal concentrations reduced the biological fitness of the F_0 generation of melon aphids. Moreover, the longevity and fecundity were decreased significantly at both LC_5 and LC_{15} concentrations,

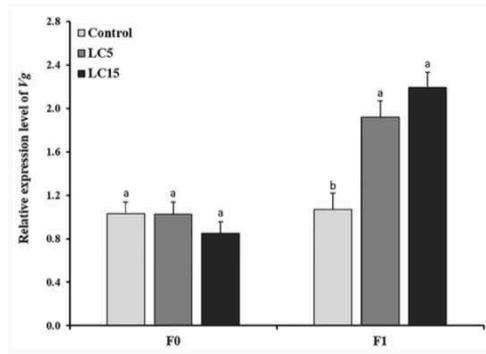


Fig. 4. Relative expression levels of vitellogenin gene in F0 and F1 generation *Aphis gossypii* descending from the parent (F0) at 72 h after the exposure with LC5 and LC15 of acetamiprid. The relative expression level is expressed as the mean (\pm SE) with the control as the calibrator. Different letters above the error bars indicate significant differences at $P < 0.05$ level (one-way ANOVA followed by Tukey's HSD test).

as compared to the control group. Similar results have also been reported for other neonicotinoid insecticides, e.g., sublethal concentrations of nitenpyram decreased the reproduction and longevity of a directly exposed F₀ generation of *A. gossypii* (Wang et al. 2017). Similarly, the adult longevity and fecundity of *A. gossypii* were reduced significantly, when exposed to LC₁₀ and LC₄₀ of cycloxyaprid (Cui et al. 2018). In addition, the longevity of *A. gossypii* was reduced, as compared to the control, after exposure to LC₄₀ and LC₁₀ of cycloxyaprid (Yuan et al. 2017). All these results show that, in addition to direct mortality induced by insecticides, the population growth of surviving individuals was also greatly affected.

The sublethal effects of insecticides in directly exposed aphids can either be negative, such as for *A. gossypii* exposed to cycloxyaprid and sulfoxaflor (Chen et al. 2016; Cui et al. 2018), or positive, e.g., sublethal concentrations of acetamiprid and imidacloprid significantly increased the nymphal duration and fecundity as well as mean generation time (T) and gross reproductive rate (GRR) in *M. persicae* (Ayyanath et al. 2013; Sial et al. 2018). Similarly, a higher net reproductive rate (R_0) of F₁ and a shorter mean generation time (T) of F₂ were observed in *M. persicae* treated with LC₂₅ of flupyradifurone (Tang et al. 2019). Furthermore, beta-cypermethrin and imidacloprid increased fertility in *Aphis glycines* Matsumura (Hemiptera: Aphididae) at sublethal concentrations (Qu et al. 2015; Qu et al. 2017). This bi-phasic phenomenon is generally characterized by low-dose induction and high-dose inhibition following exposure to stress, i.e., hormesis may have stimulatory effects in life

history traits of individuals (Rix et al. 2016). Further, hormesis may favor the population growth of insect pests, resulting in pest outbreaks or the development of insecticide resistance (Guedes & Cutler 2014). Such events occur due to the low concentrations of insecticide involved in the production of enzymes that detoxify xenobiotic compounds or increase mutations that are linked with resistance (Rix et al. 2016). Therefore, the immense application of acetamiprid and its sublethal effects must be determined.

In this study, the developmental duration of the first, second, third and fourth instars of F₁ melon aphids were prolonged significantly after exposure of the parental generation (F₀) at LC₅ and LC₁₅ of acetamiprid. Furthermore, the longevity and fecundity of the F₁ generation of melon aphids were significantly increased at both acetamiprid concentrations. Previously, transgenerational hormesis in *A. gossypii* was reported after short-term exposure to nitenpyram at low lethal and sublethal concentrations (Wang et al. 2017). Meanwhile, in *M. persicae*, stimulatory effects were observed on the duration of pre-adult and adult reproductive days, and the reproduction of the F₁ generation, when treated with LC₂₅ concentration of flupyradifurone (Tang et al. 2019). The increased population and survival rate of *M. persicae* have also been documented with sublethal concentrations of imidacloprid, azadirachtin and azinphosmethyl (Janmaat et al. 2011; Rix et al. 2016). Moreover, sublethal and hormesis effects of beta-cypermethrin and imidacloprid have been studied in *A. glycines* (Qu et al. 2015; Qu et al. 2017). The transgenerational hormetic response after exposure to low concentrations could be linked to the fact that

aphids treated with low concentrations of insecticides need long-term nutrient enrichment and mass reproduction to cope with stressful environmental conditions (Sial et al. 2018). Another potential mechanism for this extended development after low-dose exposure might be the potential uptake of acetamiprid by the progeny through the mother's body (Ayyanath et al. 2013). The developing embryos may have received a smaller amount of insecticide from the exposed mother, which resulted in a hormetic response in the F_1 generation (Wang et al. 2017).

The demographic parameters, including intrinsic rate of increase (r), finite rate of increase (λ) and net reproductive rate (R_0), were increased significantly by the LC_5 of acetamiprid. However, the intrinsic rate of increase (r) and the finite rate of increase (λ) were reduced by the LC_{15} of acetamiprid, compared to control. Our results are consistent with a previous study, in which higher demographic indexes were calculated for the progeny of *A. gossypii* treated for 72 h with LC_{10} and LC_{50} concentrations of nitenpyram, as compared to the control group (Wang et al. 2017). Similar results have also been reported in *M. persicae* when treated with 0.25 μg imidacloprid L^{-1} , compared to the control (Rix et al. 2016). The population characteristics of *A. glycines*, such as net reproduction rate (R_0), intrinsic rate of increase (r) and finite rate of increase (λ), were significantly higher than that of the control after treatment with sublethal concentrations of beta-cypermethrin and imidacloprid (Qu et al. 2015; Qu et al. 2017). The age-stage specific survival rate (s_{ij}) and age-specific survival rate (l_x) curves showed that *A. gossypii* could successfully survive and reproduce when treated with LC_5 and LC_{15} of acetamiprid, compared to the control group. However, the l_x for *A. gossypii* treated with LC_{15} was considerably higher than that of the LC_5 of acetamiprid and control, after 17 days. Similarly, the increase in F_1 fecundity in the treatments of LC_5 and LC_{15} of acetamiprid was due to the low age-stage reproductive value (v_{ij}), as compared to the control group.

Vitellogenin is a critical precursor of the egg storage protein vitellin (Vn), having a major role in the regulation of female fecundity (Tufail & Takeda 2008). Previously, sublethal effects were reported on Vg expression in 12 h old female *Chilo suppressalis* Walker (Lepidoptera: Crambidae), for instance, when treated with LC_{10} and LC_{30} of chlorantraniliprole, the fecundity and the expression level of Vg were decreased significantly (Huang et al. 2016). Similarly, a sublethal concentration of sulfoxaflor significantly reduced the AVg expression level and disturbed the development and reproduction of *Apolygus lucorum* Meyer-Dür (Hemiptera: Miridae) (Zhen et al. 2018). Additionally, the fecundity and $Nlyg$ mRNA expression were increased significantly when *Nilaparvata lugens* Stål (Hemiptera: Delphacidae) was treated with sublethal concentrations of deltamethrin and triazophos (Ge et al. 2010). In our study, the expression level of the Vg gene was increased significantly in the progeny

generation (F_1) when the parents (F_0) were exposed to low or sublethal concentrations (LC_{15} and LC_5) of acetamiprid. However, the mRNA expression of the Vg gene was not affected in the F_0 generation by either of the acetamiprid concentrations. Our results suggest that the LC_{15} and LC_5 concentrations of acetamiprid may obviously upsurge the fecundity in the progeny generation (F_1), which might be due to the increased mRNA expression of the Vg gene, allowing *A. gossypii* to better withstand subsequent stress.

Taken together, our results show that the pre-adult period, fecundity, and population parameters of F_1 *A. gossypii* were increased significantly when the parents (F_0) were exposed to low or sublethal concentrations of acetamiprid. Moreover, the increased expression level of the Vg gene in the progeny generation (F_1) might be due to the hormetic effects of acetamiprid, which ultimately upsurge reproduction in female aphids. These results suggest that exposure to low or sublethal concentrations of acetamiprid might induce hormesis in *A. gossypii*, which may lead to pest resurgence (Morse 1998). However, further study is required to determine acetamiprid-induced multigenerational hormesis in *A. gossypii* using different sublethal concentrations. Moreover, due to the greater genetic diversity in field populations, further studies are needed under field conditions, which may provide a more comprehensive evaluation of putative hormesis responses to acetamiprid. The present study is the only one to disclose the increased reproduction of melon aphids, coinciding with the induction of the Vg gene at low or sublethal concentrations of acetamiprid, across two successive generations.

Authors contributions: FU, HG, ND, and DS designed the experiment. FU and HG performed the experiments. FU, HG, and XX collected the insects. FU, HG, and YQ analyzed the data. FU, HG, ND, AMK and DS wrote and reviewed the manuscript. DS and XWG Contributed to the reagents/materials. All authors read and approved the manuscript.

Conflict of interest: The authors have declared that they have no conflict of interest.

Ethics approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Data availability: All data analyzed during this study are included in this published article.

Acknowledgments: This work was financially supported by the National Key Research and Development Program of China (2016YFD0200500) and the National Natural Science Foundation of China (31272077). We are grateful to Dr. Hsin Chi (Department of Plant Production and Technologies, NiğdeOmer Halisdemir University, Niğde, Turkey), for their statistical assistance with the two-sex life table theory used in this work.

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