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WITHIN-PLANT DEFENSE MECHANISMS AND MULTI-TROPHIC INTERACTIONS BETWEEN *SOLANUM* SPP. AND *TUTA ABSOLUTA*

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The Essence of Life

is to understand that everything is mystery

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Research highlights

- Plants developed mechanisms to improve their defenses against herbivores
- Plant resistance could be employed in Integrated Pest Management programs to increase alternative and sustainable pest control tools
- Metabolic, behavioral and genetic responses of induced and non-induced *Solanum* species were assessed
- *Tuta absoluta* is one of the most dangerous key pests of tomato plants
- The response of different *Solanum* species and tomato varieties to *T. absoluta* feeding was studied
- The studied *Solanum* species and varieties showed a variable level of suitability for *T. absoluta* larval development
- *Tuta absoluta* plant pre-infestation induced the accumulation of various defense secondary metabolites and influence the enzymatic activities
- Induced and non-induced volatiles significantly influenced the plant attraction toward *T. absoluta* and the whitefly parasitoid *E. formosa*
- At least two defense genes of tomato plants were strongly overexpressed following *T. absoluta* larvae feeding

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Abstract

The exploitation of plant-insect interactions and plant resistance to pests represents a key control option for optimizing sustainable Integrated Pest Management, thus reducing the use of insecticides. Plants are able to respond to abiotic and biotic stresses from the external environment, including insect herbivores, with complex defense responses which involve resistance and adaptation to survive. Metabolites, mechanical barriers, enzymes and volatile compounds constitute the biochemical and physical, constitutive and induced defense mechanisms of the plants. Within this context, such mechanisms were studied for the South American tomato pinworm, *Tuta absoluta* (Lepidoptera: Gelechiidae), a key pest of tomato that can although develop on several solanaceous species. Biochemical consequences of *T. absoluta* infestation and the subsequent plant suitability for the pest development in several *Solanum* species and tomato varieties were assessed. The survival, development time, weight of the pupae and eroded leaf area of *T. absoluta* (i); evaluation of secondary metabolites and volatiles produced by pre-infested plants (ii); the olfactory response of *T. absoluta* adults and of the natural enemy *Encarsia formosa* (Hymenoptera: Aphelinidae) (iii), and the expression of genes involved in plant defense responses (iv) were assessed. The results showed that there was a significant production of induced secondary metabolites that could help the studied plants to react to the herbivore attack, such as the higher quantity of glycoalkaloids known to be toxic for insects. Indeed, the bioassays on *T. absoluta* larvae development on pre-infested plant confirm this result. Moreover, the plant emission of volatile organic compounds in pre-infested plant was significantly higher than in healthy plants. This could be the reason why there was a higher attraction of these plants toward both of the pest and the parasitoid in olfactory bioassays. These aspects are supported by the results of the expression of two genes that significantly show the overexpression of the defense genes. Therefore, the plant material tested showed promising results. The implementation of some secondary metabolites or volatile organic compounds as bio-active molecules for biopesticides and as attractive or repellent substances in tri-trophic interactions could be interesting to develop. Further experiments need to be performed in field conditions, but the obtained results are important steps towards new technologies for sustainable pest control.

Keywords

Biological control, *Encarsia formosa,* Induced plant defenses, IPM, HIPVs, Metabolomics, Olfactory response, Secondary metabolites, South American Tomato borer, Tri-trophic interactions, VOCs, Volatolomics

Riassunto

La conoscenza delle interazioni pianta-insetto e della resistenza delle piante rappresenta un'opzione chiave di controllo per ottimizzare la gestione sostenibile e integrata dei parassiti e quindi per ridurre l'uso degli insetticidi. Le piante sono in grado di rispondere agli stress abiotici e biotici dell'ambiente esterno, inclusi gli insetti erbivori, con complesse risposte di difesa che comportano resistenza e adattamento per sopravvivere. Metaboliti, barriere meccaniche, enzimi e composti volatili costituiscono i meccanismi di difesa biochimici e fisici, costitutivi e indotti delle piante. In questo contesto, è stata studiata la Tignola del pomodoro, *Tuta absoluta* (Lepidoptera: Gelechiidae), un insetto chiave del pomodoro che può anche svilupparsi su diverse altre specie appartenenti al genere *Solanum*. Sono state valutate le conseguenze biochimiche dell'attacco di *T. absoluta* e i successivi effetti sullo sviluppo dell'insetto in diverse specie del genere *Solanum*. La sopravvivenza, il tempo di sviluppo larvale, il peso delle pupe e l'area fogliare erosa da *T. absoluta* (i); la valutazione di metaboliti secondari e di composti volatili delle specie del genere *Solanum* pre-infestate (ii); la risposta olfattiva di adulti di *T. absoluta* e del nemico naturale *Encarsia formosa* (Hymenoptera: Aphelinidae) (iii) e l'espressione di geni coinvolti nelle risposte di difesa delle piante (iv) sono stati valutati. I risultati evidenziano una significativa produzione di metaboliti secondari indotti che potrebbero aiutare le specie studiate a reagire all'attacco del fitofago. Ad esempio, la maggiore quantità di glicoalcaloidi è nota per essere tossica per gli insetti. In effetti, i saggi biologici sullo sviluppo delle larve di *T. absoluta* nelle specie pre-infestate, confermano questo risultato. Inoltre, l'emissione dei composti organici volatili delle diverse specie pre-infestate era significativamente superiore a quella delle piante sane. Questo potrebbe essere il motivo per cui nei bio-saggi olfattometrici vi era una maggiore attrattività per le piante pre-infestate rispetto a quelle sane sia per il fitofago che per il parassitoide. Questi aspetti sono supportati dai risultati dell'espressione di due geni che mostrano significativamente la sovra espressione dei geni di difesa nelle piante indotte. L'implementazione di alcuni metaboliti secondari o composti organici volatili come molecole bioattive per i bio-pesticidi e come volatili attrattivi o repellenti nelle interazioni tri-trofiche sarebbe interessante sia dal punto di vista delle conoscenze di base che per i risvolti applicativi nella gestione sostenibile dei fitofagi. Ulteriori prove in condizioni di campo sono necessarie, ma i risultati ottenuti rappresentano un'ottima base per lo sviluppo di nuove applicazioni per un controllo sostenibile dei fitofagi.

Parole chiave

Controllo biologico, Controllo integrato, Difese delle piante indotte, *Encarsia formosa*, HIPVs, Interazioni tri-trofiche, Metaboliti secondari, Metabolomica, Risposta olfattiva, Tignola del pomodoro, COVs, Volatolomica

General introduction

Plant defenses as sustainable and integrated control tool

Plants have developed, during their evolution, different mechanisms to improve their defenses against herbivore. Three major defense mechanisms are recognized to contribute to resistance against arthropods: antibiosis (e.g., reduced fecundity, longevity, or survival); antixenosis, which interacts with the behavior of the pest, and tolerance, which refers to plant recovery following an attack. Plant basal resistance against arthropods is a combination of these three defense mechanisms (Agut et al. 2018). Plants usually employ multiple lines of defense concurrently, rather than relying on individual defense mechanisms. This generates possible interactions (both positive and negative) between different defense strategies (Pappas et al. 2017). For example, the attack of herbivores involves the potential presence of secondary metabolites and at the same time the plants can also produce volatile compounds, such as VOCs (Volatile Organic Compounds) and HIPVs (Herbivore-Induced Plant Volatiles) that would positively influence the activity of some natural enemies of the herbivores that have elicited this resistance response, as well as other pests (Holopainen & Blande 2013).

Cultivated tomato is highly susceptible to pests, for this reason current research is aimed at identifying the traits involved in the tomato resistance pathway, in order to increase the use of plant resistance in programs of IPM (Integrated Pest Management) (Thomas & Waage 1996; Smith & Clement 2012). Research on wild tomato species is further ahead and some traits of resistance have been identified. For example, they include the density of glandular trichomes, that produce acyl sugars and terpenes with deterring activity toward female oviposition and toxicity for the larvae (Sridhar et al. 2019; Boncan et al. 2020). However, increasing plant defenses could represent a negative consequence and toxicity to other insects including natural enemies of pests (Rausher 2001).

More interesting may be the use of tomato plant defense products as tools of integrated control, such as the use of HIPVs in greenhouses as natural attractive to natural enemies (Turlings & Erb 2018). Or the use of molecules, compounds of the defense metabolism of tomato plants, as a ready-to-use active substance (Pulga et al. 2020). Or the use of more resistant and less susceptible varieties that already allow a lower preference by insects (Painter 1958).

Biological systems

In this thesis, several aspects of the plant defense mechanisms triggered by the feeding by larvae of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) were investigated. In details, crops of the *Solanum* genus, including tomato and eggplant, were used in the experiments. Moreover, to find new sustainable control strategy against insects, the relationship between these *Solanum* species and the herbivore *T. absoluta*, a key pest of tomato plants, was evaluated. Finally, the potential multitrophic effects of *T. absoluta* feeding was assessed on the orientation of an important natural enemy of the solanaceous crops, the parasitoid *Encarsia formosa* Gahan (Hymenotpera: Aphelinidae).

Solanum species

About 1,400 plant species with many morphological and phenological differences belong to the *Solanum* genus. Most, but not all, of these species are native to South America and over time spread throughout the world (Zhang et al. 2013). The most cultivated *Solanum* species worldwide with relevant economic interest are *S. lycopersicum* and *S. melongena*, respectively tomato and eggplant (Díez & Nuez 2008). Today, *Solanum* plants are widely grown in the world because of their taste, color, flavor, and nutritional properties (Heuvelink 1999). For this reason, the area covered by greenhouses of tomato is increasing in many areas of the world (Atherton & Rudich 2012). Overall, the tomato crop has a huge importance with a world yearly production of about 180 million tonnes in 2019. In details, in Italy a production of 5 million tonnes with 91.000 ha of soil cultivated to tomato crops (FAOSTAT). In the south-eastern part of the Sicily (the southernmost region of Italy), in the last decade, the area intended for protected crops has doubled, with a current area of protected crop of about 5700 ha, of which around 65% is represented by tomato (ISTA 2016, Lo Piccolo & Todaro 2021).

For the economic interest of these species many researches were focused on *Solanum* species as experimental models (Dong et al. 2020 Yang et al. 2020; Thomine et al. 2020). In many frameworks the *Solanum* species were evaluated in experimental studies to improve crop production, for new plant genetic traits and the constitution of resistance varieties, to resist to pests and to decrease the susceptibility to disease (Naselli et al. 2016; Pérez-Hedo et al. 2021). The exploitation of many *Solanum* species could give an important contribution to modern agriculture.

Tuta absoluta

Tuta absoluta is one of the most dangerous pests of tomato and also of a few other plant species, which are not necessarily phylogenetically related (Bacci et al. 2021; Silva et al. 2021). This pest is native to South America (very likely to Peru) and it was reported for the first time in Europe in Spain in 2006, and in 2008 in Italy (Tropea Garzia et al. 2012; Desneux et al. 2022). Afterwards, it spread rapidly throughout Afro-Eurasia and became a major threat to world tomato production (Santana et al. 2019).

The adult of this pest has dimensions of about 5-6 mm and a wingspan of 10-13 mm. It has a grayish color with beige nuances, but the most peculiar feature is the fringing of the wings. The egg is elliptic-subcylindrical in shape, with rounded ends and bright white or slightly yellowish in color to the deposition to tend to brownish towards maturity. The larva evolves through 4 stages (L1, L2, L3, L4) from a size of 0.4 mm to 7-8 mm at maturity. The pupae have dimensions of approximately 3.8-4.5 mm in length and 1.3- 1.5 mm in width. It is freshly formed green but immediately tending to more and more intense hazelnut, then brown when ripe (Fig.1). With temperatures of 30° C the cycle closes in about 1 month, while a temperature of 15° C the whole cycle lasts about 3 months (Sannino 2012). The damage is caused by larvae that erodes the leaf mesophyll and the fruits, damaged them irreversibly (Biondi et al. 2018). Indeed, when the *T. absoluta* attack is not controlled in protected crops and the tomato plant have a high percentage of damaged plant parts, the production is compromised.

As a consequence, it is necessary to prevent the pest damage by integrating several tools (Desneux et al., 2022). Among the many natural enemies of this pest, mostly general predators and wide host range parasitoids, zoophythophagous Mirids have shown the highest efficacy (Zappalà et al., 2013; Biondi et al., 2018). Among these, *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae) could have an important role in integrated pest management alone or combined with the use selective insectcides (Soares et al. 2019; Desneux et al. 2022). The employment of pheromones for the monitoring of the pest population and to use the mating disruption and the attract and kill techniques are also considered valid alternative to insectcides (Caparros et al. 2013; Urbaneja et al. 2013). The latter aspect, is of paramount importance because, even thought the use of synthetic insecticides could help to control *T. absoluta*, this pest has the tendency to develop insecticide resistance (Guedes et al. 2019). Finally, among the preventive strategies, the use of resistant varieties is a proming strategy (Maluf et al. 2010; Sohrabi et al. 2016; Hamza et al. 2018).

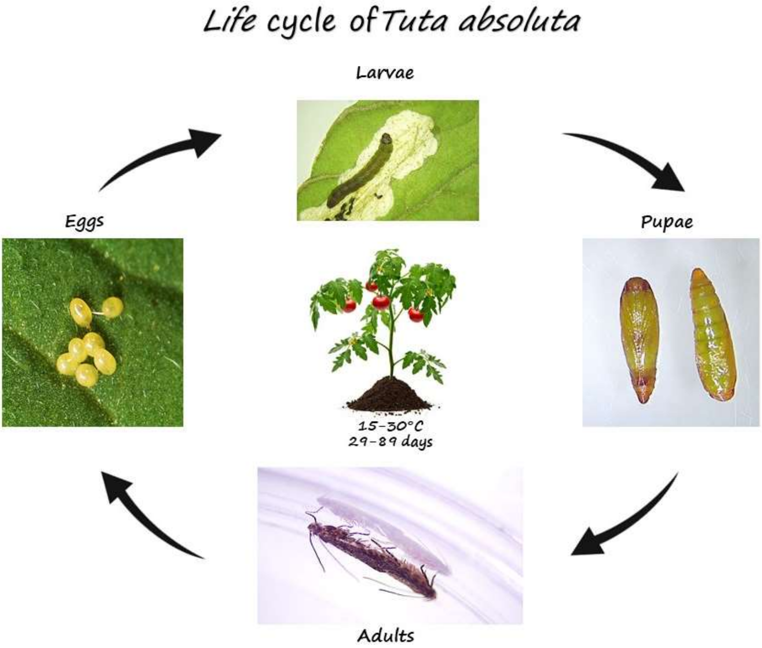

***Figure 1** – Main steps of the* Tuta absoluta *life cycle*

In this framework, *T. absoluta* interfere with tomato plant in an active way inducing plant to react and defend themself. Egg oviposition, chewing activity of the larvae launch a chain reaction of tomato plant defense developing highly specific and effective mechanisms (Hamza et al. 2018). In the tomato-*T. absoluta* interactions it is clear, thanks to the many studies recently carried out, that tomato plant is able to activate/repress the regulation of genes involved in cuticle formation and cell wall strength, but also genes involved in enzymatic activity and in the production of chemical compounds and secondary metabolites (D'Esposito et al. 2021). Specifically, defense compounds such as jasmonic acid, salicylic acid, chlorogenic acid, aminobutyric acid, total phenols, etc., as described by de Falco et al. 2019 and by Chen et al. 2021, are activated by the attack of *T. absoluta* and negatively affect the development of this pest.

Encarsia formosa

Encarsia formosa is an active parasitoid of pupae of at least 15 hosts of greenhouse aleyrodids (Rumei 1991). It is used in sustainable field pest control strategies since 1920 (Hoddle et al. 1998).

This species is distributed worldwide but its origins is uncertain. Probably, for its similar traits to *E. luteola* Howard, it is possible to attribute its origin to the Western Hemisphere (Hoddle et al. 1998). The *E. formosa* female has dimensions of about 0.6 mm in length and 0.3 mm in breadth. The head is brown and the thorax in black with yellow sides. A distinctive morphological character is the bright yellow and shining abdomen with an evident ovipositor visible at the end of the abdomen (Speyer 1927). The male is quite different only for the abdomen color that is dark brown. *Encarsia formosa* is mainly parthenogenetic and, for this reason, male occurs only if there is a high ratio parasitoid/host (Gerling 1966).

Encarsia formosa, due to its adaptive capacity and its easy-to-use in laboratory conditions, is a useful laboratory model to be tested in many contexts (Walia et al. 2021; Pérez-Hedo et al. 2021). Thanks to its biology and behavior, *E. formosa* is more attracted to locate its hosts by chemical components and secondary metabolites, especially volatiles, produced by induced plants by herbivores than healthy plants (Chen et al. 2021). For this reason, it could be interesting testing and evaluate plant resistance mechanisms involved in pest-natural enemy interactions to increase the activity of this natural enemy in a IPM system.

Aim of the thesis

The main aim of the thesis was to identify the defense mechanisms involved in the resistance of *Solanum* plants following the attack of larvae of *T. absoluta*. In induced and non-induced plants, the secondary metabolites, the enzymatic activities, the expression of plant defense genes and the volatile compound accumulation in interaction with *T. absoluta* and a natural enemy *E. formosa* were investigated. The main focus was to demonstrate how *Solanum* plants could overcame the presence of the pest with metabolomic, enzymatic and volatolomic responses. Moreover, how these responses are involved in a multi-trophic communication between plant-pest and plant-natural enemy were assessed. The results of this thesis improve the gap on the exploration of the resistance traits involved in *Solanum* species / varieties defense mechanisms and contribute to investigate new pest control tools. They could be employed in IPM programs as alternative and sustainable management methods, in order to optimize the natural defense of *Solanum* plants and to ideally implement them in an applied perspective.

Outline of the thesis

Chapter 1: Plant defenses for enhanced Integrated Pest Management in tomato (Review article)

This review article explains how plants developed a series of defense mechanisms to counteract the attack of herbivores. These can impact on food-webs at various trophic levels, in both natural and managed ecosystems, e.g. crops. The biochemical and ecological bases behind these processes are reviewed by highlighting the differences in direct and indirect, constitutive and induced defenses. In Integrated Pest Management (IPM), several pest control tools are applied in an economically-sound way in order to increase the crop resilience and reduce reliance on synthetic pesticides. Plant resistance is thus a crucial aspect of preventive pest control strategies in several agroecosystems, including tomato. In this context, we review the current literature dealing with the physiology and biochemistry of tomato plants in terms of metabolite pathways and multi-trophic interactions. We also describe recent advances in plant defense-based control tools obtained by studying the multi-trophic interactions between pests and plants in the tomato system.

Chapter 2: Plant suitability to the South American tomato pinworm and pest-induced defenses in wild and cultivated **Solanum** *species*

This study is focused on the evaluation of various *Solanum* plant suitability and pest-induction defense mechanisms by a key tomato pest, the South American tomato pinworm, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). One of the most dangerous invasive pests, particularly across the Afro-Eurasian continent. It causes drastic tomato yield losses owing to its leaf-mining activity and by fruit infestations. *Tuta absoluta* has been historically subjected to chemical control since the seventies in South America, however this strategy led to the emergence of multi-resistant populations. As a consequence, alternative control strategies have to be prioritized and, among them, the use of resistant varieties could represent a valid sustainable control strategy. However, despite the multiple constitutive and induced processes that can mediate tomato plant defenses against pests in wild and cultivated tomatoes, the commercial development of *T. absoluta*–resistant tomato cultivars are in progress. Within this context, in this study the suitability of several tomato varieties and *Solanum* species as feeding substrate for larvae of *T. absoluta*. Twelve species / varieties were chosen and it was assessed the potential for *T. absoluta* larvae to elicit systemic plant defense mechanisms.

Results showed a strong diversity in plant suitability for the development of *T. absoluta* larvae in terms of larval survival, larval development time, weight of the pupae and eroded leaf area. The results suggest that few species have potential for induced plant defense, because *T. absoluta* larvae developing on leaves cut from pre-infested plants suffered a higher mortality and fed less than those feeding on leaves cut from healthy plants. Such results suggest a possible direct antibiosis and antifeedant activity of induced allelochemicals on *T. absoluta* larvae.

The results of this article represent the first bases for selecting resistant/tolerant *Solanum* species. Moreover, studies at the multi-trophic level, i.e., including *T. absoluta* natural enemies, should be performed prior promoting the more tolerant species into integrated pest management programs.

Chapter 3: Tri-trophic interactions through plant volatile induction and expression of defense genes elicited by **Tuta absoluta** *larvae in* **Solanum** *spp.*

In this study, it was assessed how plants are able to defend themselves from multiple external factors, with which they interfere, and which trigger complex defense responses. Insect herbivores can be a biotic external factor that contributes to these responses and, in this framework, Volatiles Organic Compounds (VOCs) and Herbivore Induced Plant Volatiles (HIPVs) are important plant defense responses to allow plant-plant and plant-natural enemy communications. Insects and natural enemies detect their host or prey with many signals and plant volatiles are probably the most useful and complex to use, but unfortunately it is not easy to understand properly how they affect pest and natural enemy attraction and behavior. For these reasons, in this study, olfactory bioassays and gene expression analyses of induced and not induced *Solanum* varieties by insects could give an explanation to understand how plant defenses can influence pest and natural enemy attraction and behavior. Moreover, the various volatile components, induced and non-induced by the larvae of *T. absoluta* (Lepidoptera: Gelechiidae), of the different *Solanum* varieties tested were also analyzed with Gas Chromatography – Mass Spectrometry (GC-MS). More in details, plants of different *Solanum* varieties induced and non-induced by *T. absoluta* larvae were tested in a two-way olfactometer (Y) to evaluate the attractive or repellent response of adults of *T. absoluta* and of adults of the natural enemy *Encarsia formosa* (Hymenoptera: Aphelinidae). Furthermore, to highlight the metabolic defense alterations of the plants exposed to the larvae attack, the analysis of the expression of two genes involved in the defense mechanisms of tomato plants, PR I-II and TPS20, for the production of salicylic acid and β-phellandrene respectively, was carried out.

The results showed that there was a significant increase in the production of induced volatiles. This explains the pest and parasitoid olfactory responses that differ among induced and non-induced *Solanum* varieties. The results of the gene expression analyses provided an important evidence of defense induction in the plants. These results are useful for understanding plant defense mechanisms. Moreover, they can provide volatile compounds involved in the defense of plants that could be used in integrated pest control, for example through attractive dispensers to enhance the biocontrol services of natural enemies and in multi-trophic relationships.

Appendix A: Other scientific manuscripts

- Silica-Microencapsulated Orange Oil for Sustainable Pest Control

- Nanostructured lipid carriers of essential oils as potential tools for the sustainable control of insect pests

In these articles it was investigated the use of new bio-active compounds, such as essential oils (EOs), formulated in microcapsule of sol–gel and water, in the first manuscript, and in nanostructured lipid carriers (NLCs), in the second manuscript, to control pests. I actively participated to various stages of these research activities during the PhD course and I am co-author and author of these manuscript.

Appendix B: Contribution to Congress proceedings

In this section there is a collection of conference contributions as speaker carried out during the PhD course.

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1. Chapter 1: Plant defenses for enhanced Integrated Pest Management in tomato (Review article)

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Plant defenses for enhanced Integrated Pest Management in tomato

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1.1 Plant defenses within IPM in tomato crops

Integrated Pest Management (IPM) is based on the application of sustainable pest control combining different tools (Ehler 2006; Santoiemma et al. 2020; Gugliuzzo et al. 2021). It is a control strategy in which biological, chemical, genetic, physical and agronomic control methods are combined to pursue stable long-term pest control (Larkin 1989; Tait et al. 2021; Desneux et al. 2022). The implementation of an IPM involves three key elements: a) multiple tools used in a compatible way, for example combining pheromone-based methods and the use of natural enemies; b) prevention of high pest densities; c) conservation of biodiversity to increase ecosystem services.

In the context of IPM, plant resistance to insect pests plays a key role when designing and implementing sustainable plant protection protocols (Kennedy 2008). Enhancing plant defenses can contribute to IPM combined with rational fertilization, biological control and cultivation methods (Gharekhani & Salek-Ebrahimi 2014; Blazhevski et al. 2018). Influencing and enhancing the plant defensive profile, in which the plant has to pay a cost (i.e., a decreased above ground growth and reduced flowering as consequences of the herbivore attack), can impact insect pests and their natural enemies in commercial tomato crops (Li et al. 2018; de Oliveira et al. 2019). Tomato has been considered a model plant to characterize the defense mechanisms for many other crops. Thus, IPM seems to be a key approach to reach sustainable production in tomato, thereby preserving environmental and human health.

Here, we review the current knowledge on plant defenses and their potential impact on tomato pest control packages focusing on the trophic interactions involving tomato and its invasive pests which are causing extensive damage to several tomato production areas worldwide (Biondi et al. 2018; Mansour et al. 2018; Han et al. 2019a; Rostami et al. 2020).

1.2 The co-evolution of plant-arthropod interactions

Plant responses to insect herbivory, developed during plant evolution, involve various defense and/or tolerance mechanisms (Ehrlic & Raven 1964). Plants react to herbivores by activating a cascade of resistance mechanisms to defend themselves. These include the modification of the phytohormone profile, thus triggering direct and/or indirect defenses (Thaler et al. 2002; Kant & Baldwin 2007).

Plant defenses tend to reduce the fitness of herbivores by reducing their survival and reproduction. Thus, plants and insects may co-evolve, where co-evolution is defined as the process of genetic adaptations in a reciprocal way (Ton et al. 2009; Mithöfer et al. 2012; Dicke & van Loon 2014). When an herbivore successfully evolves to overcome the plant defense mechanisms, it might become a potential threat and it imposes a selective pressure on plants that results in the evolution of herbivore-specific induced defense mechanisms (Bergelson et al. 2001; Dodds & Rathjen 2010). Similarly, when a plant adapts to counteract the attack of an herbivore, it has the potential to be a resistant host (Jongsma & Bolter 1997).

The suitability of the plants for the herbivores that feed on them, the insect herbivore species in interaction with the plant and the gene modifications in plant and insect defense traits are three important aspects in the insect-plant co-evolution (Rasmann & Agrawal 2009; Liu et al. 2017). In most cases, co-evolution involves a compromise in which both the host and the herbivore survive and develop sub-optimally due to considerable metabolic costs (Gatehouse 2002). Organisms continuously evolve to compete, such as in a game of evolutionary ping pong (Arora et al. 2012). In particular, evolution depends on the frequency with which the attack occurs and, above all, on the behavioral and genetic responses of the insect (Berenbaum &

Zangerl 1998; Kant et al. 2008).

1.3 How plants defend themselves

Identifying and classifying the ecophysiological responses of plants is a key to improve their natural defenses (Ehrlic & Raven 1964). Plant defenses can be classified as constitutive and induced, although often the metabolites implicated are the same or similar (Duffey & Felton 1991; Wittstock & Gershenzon 2002). Constitutive defenses include products, structures and compounds of the primary and secondary metabolism that are involved in plant defenses regardless the threat of an herbivores (Hanley et al. 2007; Bar & Shtein 2019). By contrast, induced defenses are activated following an herbivore attack, or other biotic and abiotic stresses, and include products, structures and compounds of the secondary metabolism of the plants subsequently primed (Arimura et al. 2005; Kersch-Becker & Thaler 2019). The response of plants to stresses is a combination of many reactions and mechanisms that indirectly and directly influence their interactions with the ecosystem, even at the multi-trophic level and often with long-term consequences (Mithöfer & Boland 2012; Stam et al. 2014). Both constitutive and induced defenses can be direct and indirect. The direct ones influence the performance and development of the herbivore, for example, the accumulation of toxins or the thickening of cell walls (Chen 2008; Yang et al. 2020; Lin et al. 2020). Indirect defenses manipulate (i) the behavior of predators or parasitoids of the herbivore by attracting them to the infested plant; and/or (ii) of neighboring plants by warning them through alarm signals (Coppola et al. 2017). This kind of signaling is mediated by herbivore-induced plant volatiles (HIPVs) that attract the natural enemies toward the infested plant and/or prime pre-infestation defense mechanisms in nearby healthy plants (Fig.2.1) (Kessler & Baldwin 2002; Pérez-Hedo et al. 2017; Mithöfer et al. 2018). In crop protection, combining all these aspects is crucial for sustainability. However, until quite recently, most mechanistic studies have investigated the relationship between a single pest and a single type of defense-related factor or response. In fact, they have ignored the presence of other defense factors such as those related to complex food webs including concomitant multiple pest attacks (Stout et al. 1999; Stam et al. 2014).

1.3.1 Constitutive defenses

Constitutive defenses exist in plants independently of any herbivore attacks (Goyal et al. 2011). They include structural, mechanical and chemical mechanisms (Dussourd & Denno 1991; Bonaventure et al. 2011). These can be independent from each other or can act synergistically, such as glandular trichomes and their secretory canals, which combine a structural defense and a secretory capacity as mechanical and chemical defense mechanisms (Glas et al. 2012; Wang et al. 2021).

1.3.1.1 Structural and mechanical defenses

Morphological barriers of the plant, e.g., thorns, can prevent feeding by large herbivores while the thickening of the walls, for example, can impair feeding by small herbivores (Wheeler & Krimmel 2015; Mitchell et al. 2016; Jacob et al. 2020). Bitew (2018) found that wild tomato species with high density of glandular and non-glandular trichomes, such as *S. habrochaites* and *S. pennellii*, have an important potential for resisting to *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). These species and/or these traits could thus be included in future tomato breeding programs, e.g., by identifying the genetic basis involved in the production and density of mechanical barriers. However, this might result in further challenges because trichomes in tomato plants can strongly compromise the establishment of predatory arthropods (including mites and flower bugs) and, thus, the ecological services they provide (Salehi et al. 2016; Paspati et al. 2021).

1.3.1.2 Chemical defenses

Constitutive chemical defenses (e.g., tannins, resins) can be toxic, repellent or antifeedant and can impact on the development of the herbivores by altering the resistance, the fecundity, and the digestive capacities. The toxicity or repellency of these chemical compounds also depend on how the plant stores them. For example, large amounts of toxicants are released by the plant only once the herbivore breaks down specific plant structures by feeding or just moving on the plant, such as resin ducts or glandular trichomes (Dearing et al. 2005; Zhou et al. 2015; Yousaf et al. 2018; Giordano et al. 2020). Fractions of tomato trichome extracts can affect predatory mite survival in a concentration-response manner (Paspati et al. 2021).

Kennedy (2003) reported that the presence of phenolic compounds, e.g., chlorogenic acid and rutin, in tomato leaves, may inhibit the growth of larvae of two noctuid pests, *Spodoptera exigua* (Hübner) and Helicoverpa zea (Boddie). Phenolic compounds are typically synthetized in the green parts of the plants and are considered as constitutive defense chemicals (Harborne 1979). When incorporated into an artificial diet, they inhibit larval development in a dose-dependent manner (Isman & Duffey 1982; Kennedy 2003).

1.3.2 Induced defenses

External factors that interfere with the plant, such as herbivores, can trigger plant defenses (i.e., signaling cascades) which make the plant synthesizing specific compounds, such as secondary metabolites, and trigger changes in plant physiology. This hampers the fitness, the survival, the development and the fecundity of the feeder (Kant et al. 2004). The success of plant defenses is mediated by a prompt and specific identification of the herbivore. Santamaria et al. (2018) reported how the different feeding mechanisms of herbivores enable the plant to recognize them, through specific plant receptors (PRRs, pattern recognition receptors), damage-associated molecular patterns (DAMPs) and herbivore-associated molecular patterns (HAMPs), and respond accordingly by triggering short-term and long-term defense mechanisms.

Herbivore attack may affect metabolomic content in plant tissues. As described by Pappas et al. (2015) and Pérez-Hedo et al. (2018) in tomato plants as response to the attack of *Macrolophus pygmaeus* (Rambur) and *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae), induced defenses are primed both locally and systemically. This involves different signaling pathways related to systemin, jasmonic acid (JA or jasmonate) and salicylic acid (SA).

In this context, it was demonstrated that trichome development is correlated with the abundance of jasmonic acid (Boughton et al. 2005; Tian et al. 2012). Paudel et al. (2019) showed that the development of both glandular and non-glandular trichomes in tomato are induced as a result of herbivore damage.

Pérez-Hedo et al. (2015a) investigated the implications of different phytohormones involved in tomato plant defenses in response to N. tenuis punctures. They evaluated the production of induced defense mechanisms, such as the production of HIPVs playing a key role in tri-trophic interactions (Turlings & Erb 2018). These mechanisms are crucial to understanding the evolution of plant-natural enemy relationships (Sabelis et al. 2001; Stahl et al. 2018). Through plant induction, HIPVs can make the plant repellent to herbivores (Engelberth et al. 2004; Frost et al. 2008), and/or more attractive to natural enemies of pests (Pérez-Hedo et al. 2015b; Naselli et al. 2016; Ayelo et al. 2021). In this context, Conboy et al. (2020) analyzed tomato HIPVs and selected methyl salicylate (MeSA), a plant elicitor that recognizes and triggers intracellular defense signaling in the plant. They evaluated the application of this elicitor in un-infested tomato plants and noted that the *Trialeurodes vaporariorum* (Westwood) (Hemiptera: Aleyrodidae) population was reduced and that there was an 11% increase in the tomato yield.

In some cases, insects can modulate HIPV production by the attacked plant (Sarmento et al. 2011). Zhang et al. (2019) demonstrated how *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) can manipulate the defense mechanisms of the host plant community by stimulating the attacked plant to emit HIPVs that can prime SA-dependent defenses and suppress JA-dependent defenses in neighboring plants. This would make the neighboring healthy plant more suitable for the imminent attack by *B. tabaci*.

Moreover, herbivore attack may affect the polyphenol oxidase (PPO) and protease inhibitors (PIs) in plant tissues, resulting in hampered larval growth (D'Esposito et al. 2021). The larval growth of insects that feed on damaged leaves decreases and this demonstrates that tomato plants show a greater degree of resistance after herbivore attack (Escobar-Bravo et al. 2017; Hamza et al. 2018).

1.4 Signal transduction and metabolite pathways in tomato

Plant hormones play a central role for plant signaling networks as response to biotic and abiotic stresses (Robert-Seilianiantz et al. 2007). When an herbivore begins to feed on plant tissues, the damage generates a plant defense response in a generally non-specific way (Frost et al. 2008), and primary and secondary metabolites belonging to disparate chemical classes are produced by plants (Duffey & Stout 1996; Wink 2008). Nevertheless, the signals produced by herbivores can be specific and enable the plant to respond in a much more precise way.

The hormone jasmonic acid has a key role in tomato plant resistance to herbivores, mainly chewing ones. This hormone induces the transcription of genes involved in defense metabolism (Turner et al. 2002). It is produced in the signaling octadecanoid pathway and it is synthesized in the chloroplast and peroxisome from linolenic acid, which is released from plant membrane lipids (Chen et al. 2006).

Other hormones, such as salicylic acid and ethylene, participate in the signaling pathways involved in the defense mechanisms (Lorenzo et al. 2003; Zarate et al. 2007).

In tomato plants, the preliminary step in the signaling and defensive response pathway is the segmentation of the precursor prosystemin, a leaf constitutive polypeptide (Fig. 2.1). Specific enzymes contained in the saliva of herbivores can activate the catalysis of the peptides in the cell membrane leading to the transformation of prosystemin in the peptide systemin, a plant hormone (Ryan 2000; Li & Howe 2001). Thanks to systemin, that is translocated in the plant phloem, defensive signaling can become systemic throughout the plant. The defensive signal transduction activates phospholipase A2, which in turn releases linolenic acid from membrane lipids. Linolenic acid acts as a precursor for the synthesis of the octadecanoid pathway of jasmonic acid.

In the next step the pectinic component of the cell walls of plants is splitted in oligomeric polymers (oligogalacturonic acid - OGA) which are defensive proteins of tomato plants. The signal which activates expression of the wound-induced polygalacturonase gene appears to be jasmonic acid (Orozco-Cardenas & Ryan 1999), suggesting that this has an earlier effect in the signaling pathway than in oligogalacturonic acid. At the end of the signaling pathway of defensive proteins, such as proteinase inhibitors and polyphenol oxidase, hydrogen peroxide is produced and diffused in mesophyll cells through the oxidative burst, near the vascular bundles (Orozco-Cardenas et al. 2001). Once absorbed by insects, hydrogen peroxide negatively affects the permeability of the intestinal wall causing oxidative damage to the insect epithelial cells (Singh & Singh 2021).

1.5 *Alterations in the plant defensive profile and their multi-trophic outcomes*

External biotic and abiotic factors, such as for example irrigation and fertilization inputs, or the preliminary priming by other insects, or by

HIPVs from infested plants, could enhance plant resistance mechanisms, thereby offering a significant boost in sustainable tomato IPM.

1.5.1 Bottom-up approaches

The bottom-up approaches consist in exploiting the effects of variable availability in the soil of water, of minerals (e.g., potassium and nitrogen), and/or (bio)fertilizers for optimizing the defense mechanisms of the plants (Larbat et al. 2016). Due to bottom-up effects, modified defense volatiles and altered plant metabolism can affect tri-trophic interactions (Denno et al. 2002; Han et al. 2014; Coqueret et al. 2017; Shehzad et al. 2020). Salinity stress increases the osmotic potential, thus decreasing plant water availability, leaf dietary quality (Romero-Aranda et al. 2001; Manaa et al. 2011; Teklić et al. 2020), and also water availability to the larvae of herbivores. The secondary metabolism of the plant can also be modified by salinity stress, and plants can alter their defense compounds thus affecting the trophic interactions (Ballhorn & Elias 2014). In order to resist the salinity stress of plants, insects are able to modify their feeding intensity and to shorten the juvenile development time (Han et al. 2019b).

The quantity and quality of the plant watering can enhance the plant defense mechanisms thus affecting the plant resistance to herbivores (Dong et al. 2020; Gutbrodt et al. 2011; Dong et al. 2018). Lin et al. (2021) studied how lower water availability increased the levels of two tomato plant defensive proteins, trypsin protease inhibitor and polyphenol oxidase. It was shown how these resistance factors directly influence the consumption of plant tissues and the performance of *Manduca sexta* (Linnaeus) (Lepidoptera: Sphingidae) a specialist caterpillar (Lin et al., 2021). Furthermore, in conditions of water deficit, the increase in the concentration of glycoalkaloids in the tomato leaves negatively affects survival, pupal weight and larval development time of *T. absoluta* (Han et al. 2016).

Volatile organic compounds (VOCs) are affected by water limitation in tomato plants and this influenced the plants' level of attraction for *T. absoluta* and *B. tabaci* adults preferring moderately stressed and non-stressed plants, respectively (Pagadala Damodaram et al. 2021). VOCs can also be altered by temperature as demonstrated by the invasive weed *Alternanthera philoxeroides*, although this did not influence its attraction for two herbivores (Liu et al. 2021).

The quantity and the quality of nitrogen strongly limits herbivore development (Han et al. 2020, Kagata & Ohgushi 2012), thus making the concentration of nitrogen important for the survival of insects. The amount of nitrogen and metabolites in plant tissue is affected by nitrogen fertilization. Many primary metabolites, such as carbohydrates, as well as other secondary plant metabolites, such as phenolic acids, flavonoids and glycoalkaloids, are influenced by nitrogen fertilization and can impact insect development (Fritz et al. 2006; Hermans et al. 2006; Larbat et al. 2016; Coqueret et al. 2017). Tomato plants with low nitrogen input produce higher concentrations of defensive secondary metabolites, such as soluble phenolics (i.e., kaempferol-rutinoside, chlorogenic acid and rutin), because their concentration is connected positively with the carbon/nitrogen ratio in tomato tissues (Royer et al. 2013).

All these compounds are repellent and/or toxic to various insect pest taxa (Mirnezhad et al. 2010). For example, Ramachandran et al. (2020) studied the effects of nitrogen availability on the tolerance to whiteflies in four tomato varieties showing that plants grown at half the optimal quantity of nitrogen had a higher degree of resistance to whiteflies, without a decreasing yield.

Tomato plants, in conditions of nitrogen deficit limits, negatively affect the survival, pupal weight and development time of *T. absoluta* (Coqueret et al. 2017). This seems to be due to the low protein content and to the increased number of phenolic compounds and glycoalkaloids in the leaves (Larbat et al. 2016). Overall, in tomato the production of various chemical defense molecules, mainly based on carbon and less on nitrogen, reduces the nutritional value of the leaves for herbivores (Larbat et al. 2012; Royer et al. 2013). In addition, water and nitrogen quantities are positively related, and larvae of *T. absoluta* may thus suffer from a lack of nitrogen-based nutrients in drought-prone plants (Han et al. 2014).

Another macro-element that can alter the concentration of defense compounds is potassium (Trejo-Escobar et al. 2019) which has bottom-up effect potentials on the cycle of *B. tabaci* and *T. absoluta* in tomato plants. It was showed by Darwish et al. (2021) that treatment with potassium fertilizer as a foliar spray on tomato plants can have a significant impact on decreasing the density of *B. tabaci* nymphs. Sung et al. (2015) studied the low potassium input on the tomato plant and found that there is an accumulation of defense compounds in the plant tissue, such as soluble sugar and putrescine, known to be involved in defense responses of the plant (Liu et al. 2020), and a concomitant reduction of the majority of the amino acids.

Biological fertilizers have received considerable attention in the last years for their potential employment in sustainable crop protection. Beneficial microorganisms can enhance plant performance and plant defenses under different conditions, such as herbivore attack. For example, *Trichoderma longibrachiatum* modulates the expression of classes of transcription factors and of genes involved in plant photosynthesis and antioxidant defenses (De Palma et al. 2021). Megali et al. (2014) showed how the mix of beneficial microorganisms, brought to the soil, affects the yield of tomato plants and enhances the defenses of the plant from the attack of *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae). This could have been attributed to the increased presence of glycoalkaloids, such as tomatine, which influence the development of the insect.

1.5.2 Indirect interactions

Indirect interactions consist of those plant relationships with external factors, such as insects and microorganisms, which can influence plant responses and modify interactions with other members of the system, such as for example natural enemies (Stam et al. 2014). Plant responses lead to morphological or metabolic changes which can subsequently influence indirect interactions with other members of the community (Han et al. 2020). Thus, plant defense profile can be enhanced by a preliminary attack from herbivores (Poelman & Dicke 2018). The initial insect feeding can stimulate the defense pathways of the plant, thereby generating secondary metabolites, including HIPVs, which can interfere with the choice of the host by the pest and the natural enemies. For example, Pérez-Hedo et al. (2015a, b) showed that tomato plants fed by *N. tenuis* activate the jasmonic and abscisic (ABA) acid pathways making the plants less attractive to *T. absoluta* and *B. tabaci*, but more attractive toward Encarsia formosa Gahan (Hymenoptera: Aphelinidae), one of the most effective whiteflies' parasitoids.

Moreover, it was shown how the spider mite feeding on tomato plant causes an emission of HIPVs depending on the induced JA signaling. The methyl salicylate (MeSA) and 4,8,12-trimethyl-l,3(E),7(E), 1 1-tridecatetraene (TMTT) were emitted after the attack of *Tetranychus urticae* Koch (Acarina: Tetranychidae) (Ament et al. 2004; Kant et al. 2008). These volatile compounds have an important role on the indirect defenses to attract natural enemies such as *Phytoseiulus persimilis* Athias-Henriot (Acarina: Phytoseiidae) (De Boer & Dicke 2004). Similarly, Ayelo et al. (2021) found that, among the many HIPV induced by *T. absoluta*, several monoterpenes are useful to attract the zoophytophagous mirid *N. tenuis*, while (E)-β-caryophyllene was found to repel it.

Other researches are focused on the evaluation of different tomato types with different production of jasmonic acid and the attraction of natural enemies. The production of jasmonic acid has a key role for the production of defense volatile compounds to attract natural enemies. Indeed, it was analyzed by Thaler et al. (2002) how P. persimilis is less attracted to tomato types with jasmonic acid-deficient induced by the feeding of *S. exigua* than tomato types with a good jasmonic acid production.

Pérez-Hedo et al. (2021) studied how specific HIPVs, isolated from tomato plants attacked by *N. tenuis*, for example (Z)-3-hexenyl propanoate, can indirectly prime the defenses of healthy plants. In a tomato greenhouse, they tested how the selected HIPVs, constantly released by polymeric dispensers, can prime commercial tomato plant defenses for more than two months and thus reduce the attack of key pests. Indeed, these HIPVs elicited the production of secondary metabolites, the expression of jasmonic acid and salicylic acid signaling marker genes, and a large number of protein inhibitors. In addition, the levels of *T. absoluta* and *T. urticae* infestation were significantly lower in the treatment with the (Z)-3-hexenyl propanoate.

Moreover, microorganisms can also be useful for recruiting pest natural enemies indirectly. Battaglia et al. (2013) studied how the use of the plant growth promoting fungus *Trichoderma longibrachiatum* can affect the tomato-herbivore-parasitoid/predator multi-trophic system. The soil inoculation with *T. longibrachiatum* boosted the development and reproduction of *Macrosiphum euphorbiae* (Thomas) (Hemiptera: Aphididae) but also of the generalist predator M. pygmaeus. Moreover, *T. longibrachiatum*-infected plants have been shown to be more attractive towards the aphid parasitoid *Aphidius ervi* Haliday (Hymenoptera: Braconidae) and *M. pygmaeus*.

1.6 Enhanced plant defenses: an important tool for future tomato IPM programs

Enhanced plant resistance, either constitutive or induced, is and will be a key component of IPM in tomato. Therefore, to promote plant resistance to herbivores, plant resistance inducers could be used much more intensely, for example the exploitation of agents that improve protection against pest attacks by priming plant defense mechanisms could be pursued. Unlike traditional pesticides, plant resistance inducers do not directly target the insect, but hinder its development indirectly by stimulating the defense responses of the plant. Their implementation in an IPM strategy has been strongly recommended (Siah et al. 2018).

Combining plant resistance inducers with pre-infestation would also help to boost protection in plants. Esmaeily et al. (2021) described how resistance in tomato plants was induced by the foliar and root application of resistance inducers, i.e., JA and ABA, in combination with pre-infestation by *N. tenuis*. Such treatment reduced the fitness and the reproduction of *T. vaporariourum*, mainly due to an increased activity of plant enzymes and of phenolic content. Moreover, the application of other hormones as resistance inducers, i.e. salicylic acid (SA), β-aminobutyric acid (BABA), methyl jasmonate (MeJA), could amplified plant resistance (Stout et al. 1998; Jafarbeigi et al. 2021). This might suggest that increased plant enzyme activity and phenolic content when combined with pre-infestations and resistance inducers, instead of distinct and separated treatments with only inducers or only pre-infestation, will give better results on plant defense mechanisms. The enhancement of plant resistance to herbivores has also been shown for *T. absoluta*. Several wild tomato accessions have been used extensively to breed several commercial tomato lines with enhanced levels of pest resistance. For example, breeding programs using *S. galapensis*, a wild solanaceous plant, led to commercial tomato varieties resistant and/or tolerant towards herbivores, including *T. absoluta* (Snoeren et al. 2017).

Despite intensive work on these aspects and the worldwide relevance of the tomato industry, very few current tomato cultivars are tolerant to *T. absoluta*. It has been hypothesized that a low level of genetic variability has been introduced while domesticating tomato, and the consequent loss of genes controlling the production of plant defense allelochemicals, may have caused the tomato lack of tolerance to *T. absoluta* (Snoeren et al. 2017).

In this context, the use of inducers and of pre-infestation to enhance plant resistance may be a key tool for sustainable pest management programs. Induced plant defenses against insects are indeed an aspect of biochemistry, physiology and genetics of plants deeply studied worldwide and frequently proposed in IPM (Goyal et al. 2011). This means that new technologies to control herbivores are in place and the results obtained are promising (Camargo et al. 2016). Moreover, plastid-mediated RNAi technology could be a powerful tool to develop resistant cultivars against insect pests (Zhang et al. 2015). Indeed, new findings on genes in terms of herbivore resistance are likely to promote additional research in this field.

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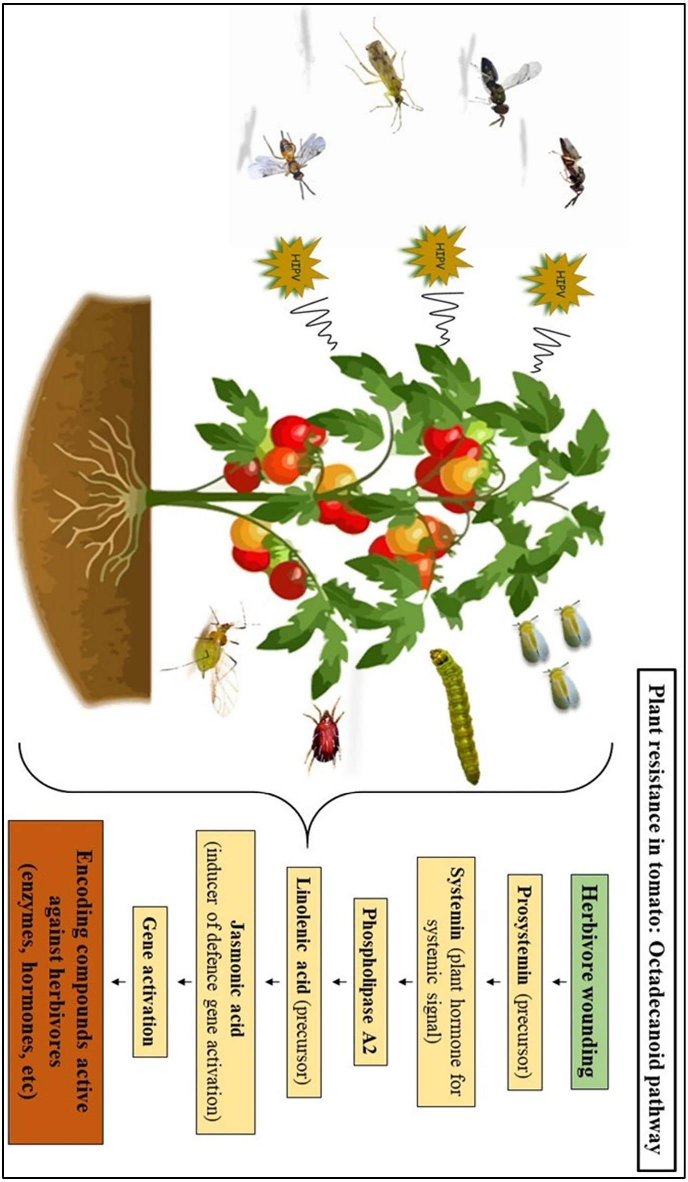
Figure legends

Figure 1.1. *Resistance mechanisms of tomato plants induced by its main herbivores involving volatile compounds (Herbivore-Induced Plant Volatiles: HIPVs) and the response of natural enemies as well as the chemical pathway to encoding active compounds against insects (enzymes, hormones, etc)*

Fig. 1.1


Plant resistance in tomato: Octadecanoid pathway
Herbivore wounding
Prosystemin (precursor)
Systemin (plant hormone for systemic signal)
Phospholipase A2
Linolenic acid (precursor)
Jasmonic acid (inducer of defence gene activation)
Gene activation
Encoding compounds active against herbivores (enzymes, hormones, etc)
HIPV
HIPV
HIPV

2 Chapter 2: Plant suitability to the South American tomato pinworm and pest-induced defenses in wild and cultivated *Solanum* species

Simona Tortorici et al., in preparation for submission to *Journal of Chemical Ecology*

Plant suitability to the South American tomato pinworm and pest-induced defenses in wild and cultivated *Solanum* species

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2.1 Introduction

Plants are able to respond to abiotic and biotic stresses from the external environment (Van der Does et al., 2017), including insect herbivores (Hilker & Meiners, 2006), with complex defense responses which involve resistance and adaptation to survive (Gatehouse 2002). Metabolites, mechanical barriers, enzymes, volatile compounds and much more constitute the biochemical and physical, constitutive and induced defense mechanisms of the plants (Smith et al. 2005; Poelman et al. 2010; Gutbrodt et al. 2012; Silva et al. 2020). One of the main purposes for modern agriculture is represented by the increase of the capacity of cultivated plants to respond even more efficiently to external factors (De Vos & VanDoorn 2013). This is currently pursued for various crops by amplifying the plant defense responses at both intrinsic, pest-induced and genetic level (Naselli et al. 2016; Vargas-Ortiz et al. 2018). The interaction plant – insect becomes of essential interest to understand which are the ways to follow to identify, characterize and increase in the plant defense mechanisms (Sharma et al. 2020).

By increasing the resistance of the plant, it will be possible to reduce the control and prevention measures on the crop for the health of the plants (Sharma et al. 2007). Thus, plant resistance is a crucial aspect of preventive pest control strategies in several agroecosystems (Hoy 2012; Miyazaki et al. 2017) and it could represent sustainable control options for optimizing Integrated Pest Management (IPM). The importance of optimized crop plant defenses could also be useful to mitigate the phenomenon of pest resistance to different active ingredients of commercial insecticides. This is the case of the South American tomato pinworm, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), which has developed resistance to the several used active ingredients (Guedes et al. 2019). Indeed, this pest has been historically subjected to chemical control since the seventies in South America (Biondi et al. 2018), but this strategy led to the emergence of multi-resistant populations (Roditakis et al. 2018; Grant et al. 2019). *Tuta absoluta* is one of the most dangerous pests of tomato (Desneux et al. 2022; Campos et al. 2017), particularly across the Afro-Eurasian continent since its first invasion in Europe in 2006 (Biondi et al. 2018), and it is able to develop on several other solanaceous species (Sylla et al. 2019; Idriss et al. 2020). The larvae feeds by feeding within the leaf mesophyll, thus producing a thin leaf mine (Biondi et al. 2018). Considering this aspect, plant defense compounds could play a crucial role in herbivore fitness and can influence the related tri-trophic interactions (Han et al. 2019).

In this context, the aim of this work was to characterize the plant defense response following the attack of *T. absoluta*, and the subsequent plant suitability for the insect itself in 12 tomato *Solanum* commercial varieties / species. Bioassays on larval survival, larval development time, pupal weight and eroded leaf area were assessed for larvae feeding in healthy and in pre-infested leaves. Then, secondary metabolites, such as polyphenols and glycoalkaloids, and enzymes involved in oxidation processes (i.e., PPO and POD) were characterized and identified. The outcomes of this research can be helpful for better understanding the bases of potential defense mechanisms induced by *T. absoluta* in cultivated and wild *Solanum* species. Results may ultimately be helpful for better designing breeding programs of new tomato varieties.

2.2 Materials and methods

2.2.1 Insect rearing

Larvae of *T. absoluta* used for the experiment were collected from the laboratory insect rearing of the Agriculture, Food and Environment Department (Di3A) of the University of Catania (Italy). The laboratory insect rearing was established in 2008 and it originated from individuals collected in many commercial tomato crops of South

Eastern Sicily. Twice a year, new individuals collected from the field were added to the colony. Individuals of *T. absoluta* were reared in *S. lycopersicum* var ‘Creativo’ as described in Zappalà et al. (2012). The colony was located in a chamber with controlled environmental conditions, as 25 ± 2 °C of temperature, 50 ± 10 % of relative humidity and L14:D10 of photoperiod.

2.2.2 Plant material

Solanum lycopersicum var ‘Creativo’ was used as host tomato plant to rear the insects, it was grown outdoor into a 10 cm diameter pots in a cage (300 × 80 × 120 cm) of insect-proof net to avoid pest infestation in natural environmental conditions.

Two types of tomato varieties were used in the experiments:

- three typical experimental model varieties, i.e., *S. lycopersicum* var ‘Better bush’® (Tomato Growers, USA), var ‘San Marzano nano’® (Bavicchi, Italy), var ‘Optima’® (Dotto, Italy) (Peterson et al. 1991; Catola et al. 2018; Alfosea-Simón et al. 2020).
- five commercial tomato varieties typical of the Mediterranean protected tomato systems: var ‘Cikito’® (Monsanto, USA), var ‘Delizia’® (Clause, France), var ‘Marinda’® (Nunhems, Germany), var ‘Rovente’® (Monsanto, USA) and var ‘Tyty’® (Syngenta, China) (Siscaro et al. 2019).

The wild *Solanum* species used in the bioassays were selected, in the case of the European black nightshade, *S. nigrum* for its high availability in the Mediterranean basin and good suitability for *T. absoluta* development (Campos et al. 2021); while, two wild tomato species *S. habrochaites* and *S. pennelli* have been selected because previous studies showed the low susceptibility of these species and the production of allelochemicals responsible for high levels of arthropod resistance (de Azevedo et al. 2003; Maluf et al. 2010). Seeds of *S. nigrum* were collected near the Di3A in Catania on July 2017; while seeds of *Solanum habrochaites* (CRB-Leg code: T300007) and *S.*

pennelli (CRB-Leg code: T300107) originated from the seed collection of the research unit of Génétique et Amelioration des Fruits et Légumes (UR1052) of the Institut national de recherche pour l'agriculture, l'alimentation et l'environnement (INRAe, France).

Finally, *S. melongena* var 'Black beauty'® (Dotto, Italy) was included in the bioassays as host's non-preferred eggplant variety (Chen et al. 2021).

Seedlings were obtained sowing in a pot (10 × 10 ×15 cm) with mixture of topsoil (Gramoflor®, GmbH & Co. KG) and expanded vermiculite (VIC, Italiana®) and were grown in greenhouse (200 × 300 × 300 cm) with a higher intensity of light. Every 15 days, the fertilizer Greenleaf 20.20.20® (Biolchim) was added in the irrigation water. Plants were used for the experiments when they had five true leaves.

2.2.3 *Assessment of the plant suitability for* Tuta absoluta *larval development*

In order to evaluate the plant suitability to *T. absoluta*, a Two-cup experimental arena (Biondi et al. 2012) was used to maintain cut *Solanum* species / variety leaves. From each healthy tested plant, the fifth leaf was cut, placed in the arena and two newly molted second instar larvae of *T. absoluta* were transferred onto the leaf. Arenas were thus kept into climatic chamber with controlled environment conditions (T: 24 ± 2 °C; RH: 50 ± 10 %; L12:D12). Larval survival and the development time, from L2 to pupae were checked daily until pupation. Then pupae were gently removed from their silky cocoon and weighted by using a precision balance (Mettler Toledo AG104). Finally, *Solanum* species / variety leaves in which the two *T. absoluta* larvae have fed were removed from the arena, placed on a document scanner (Epson® Perfection 4180 Photo) for getting a high-resolution picture in JPG format (800 dpi) of the leaf. The imagine obtained was processed for estimating the eroded leaf area, i.e., the leaf surface with *T. absoluta* mines, by using the software Adobe Photoshop® (Adobe

System Inc. 1990-2018) and a scale by a digital ruler was set as 318pixel = 10mm. With “Magnetic lasso” implement the eroded leaf area was manually selected and the data were collected. The same scale was used for all the replicates.

2.2.4 Pest-induced defense mechanisms

In order to evaluate the potential defense mechanisms induced by *T. absoluta*, we carried out a bioassay with the same modalities presented in the previous sections except for the fact that the leaf used for the experiment originated from a plant in which *T. absoluta* had previously fed. For this, the plants for the experiment had been induced by let eight second instar larvae of *T. absoluta* feeding in the third and fourth leaf of the *Solanum* plants.

In non-induced plants was stimulated the inoculation of the larvae in each third and fourth leaf to nick the trichomes with a brush (the same used for transferring the larvae in the induced plants). After three days of larval feeding for the pre-infested plants and for control and healthy plants, the fifth healthy leaf was cut from each pre-infested plant. Cut leaves from control and pre-infested plants were placed individually into the arena and two second instar larvae of *T. absoluta* were transferred. At the same time, the sixth leaf of all plants was cut and immediately frozen in liquid nitrogen and was stored in a -40 °C freezer for the enzymatic activity and secondary metabolite analyses.

Frozen leaves were then crushed until getting powder by using a mortar with a pestle in liquid nitrogen. For the extraction of the secondary metabolites the follow experimental protocol was used and it was adapted from Bénard et al. (2009). The powder of the crushed leaves (200 mg) was taken and was mixed with Methanol (100%) (1 mL) and Taxifolin (2 mg/mL) (50 μL). After one night, the samples were centrifuged (10 min / 13000 rpm) to take the liquid part. The solid part was mixed again with Methanol (100%) (1 mL) to be centrifuged (10 min / 13000 rpm) and to take the liquid part with the other. The Speed Vacuum Concentrator (Eppendorf Concentrator plus®, COUNTRY) was used to permit the total evaporation of the Methanol (100%) and to have the solid part. The induced secondary metabolites were analyzed using Ultra-Performance Liquid Chromatography coupled with Mass Spectrometry (UPLC-MS, Thermo Scientific UltiMate 3000 and Thermo Scientific LTQ XL) after the suspension of the solid part with Methanol (70%) (500 µL) and a centrifuge (10 min / 13000 rpm).

To evaluate the activities of two enzymes, i.e., Polyphenol oxidase: PPO and Peroxidase: POD, the follow experimental protocol was used and it was adapted from Stout et al. (1997). The plant leaves powder (50 mg) was mixed with a specific buffer for the extraction (500 µL) and was centrifuged (10 min / 10000 rpm / 4 °C) to take the liquid part. The Spectrophotometer Microplate Reader (Biotek Synergy HT) was used for the evaluation of the centrifuged samples (15 µL: PPO; 10 µL: POD) in a plate after the addition of a specific pre-warmed buffer (240 µL).

2.2.5 Data analysis

The statistical analysis was carried out by using the software *R* (Version 4.1.1 (2021-08-10), package “agricolae” version 1.3-5). Data on larval survival, larval development time, pupal weight and eroded leaf area for plant suitability of all the species were analyzed with one-way analysis of variance (ANOVA). For pest-induced defense mechanisms, within each *Solanum* species / variety the data were analyzed comparing induced and non-induced plant with ANOVA.

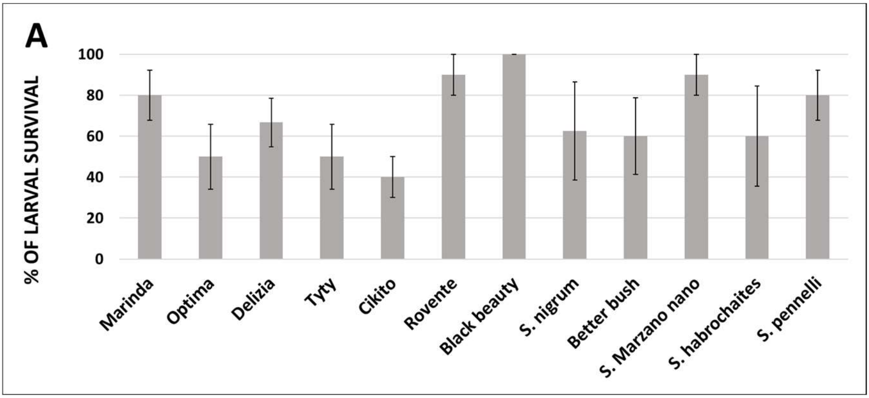
The differences in the UPLC-MS data of the secondary metabolite amount of the induced and non-induced *Solanum* species/varieties were performed with the online platform Xcms^{tm}. The non-parametric test Kruskal-Wallis comparing all the chromatograms was performed. The Student’s t-test was used to evaluate the differences between each induced and non-induced *Solanum* species / varieties in terms of enzymatic activity PPO and POD. The results were analyzed with the software Microsoft Excel® and were expressed as mean±SE.

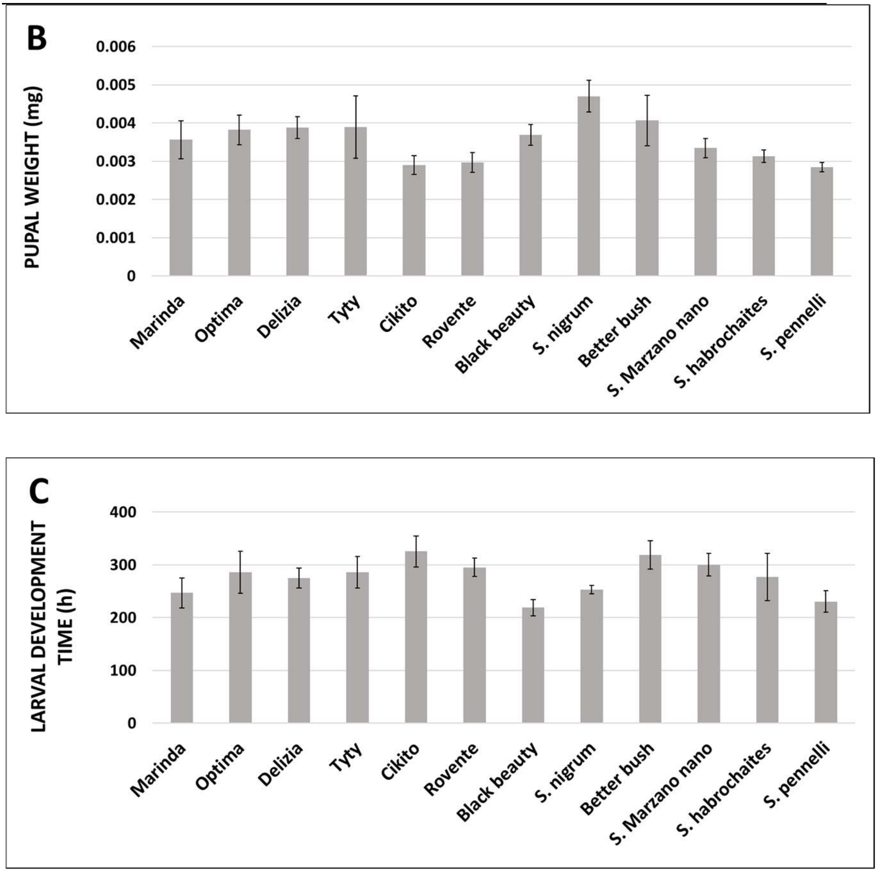
In all the analyses, the significant results were considered with a confidence level of 5% or $p \leq 0,05$.

2.3 Results

2.3.1 Plant suitability for Tuta absoluta *larval development*

The results about the analysis of plant suitability (Fig. 2.1) show a not significant difference between all the *Solanum* species / varieties for the % of larval survival ($F_{11,\,63}=1.544$; $p=0.145$), the pupal weight (mg) ($F_{11,\,55}=1.511$; $p=0.163$), the larval development time (h) ($F_{11,\,55}=1.22$; $p=0.303$) and the eroded leaf area (mm^2)/n° of *T. absoluta* larvae released ($F_{11,\,63}=0.724$; $p=0.710$). However, especially in the case of larval survival, it could be observed that there were differences between *Solanum* species / varieties. For example, the most evident one is between the eggplant var 'Black beauty' and the tomato var 'Cikito' that showed the highest and the lowest larval survival, respectively.




B
PUPAL WEIGHT (mg)
0.006
0.005
0.004
0.003
0.002
0.001
0
Marinda
Optima
Delizia
Tyty
Cikito
Rovente
Black beauty
S. nigrum
Better bush
S. Marzano nano
S. habrochaites
S. pennelli
C
LARVAL DEVELOPMENT TIME (h)
400
300
200
100
0
Marinda
Optima
Delizia
Tyty
Cikito
Rovente
Black beauty
S. nigrum
Better bush
S. Marzano nano
S. habrochaites
S. pennelli

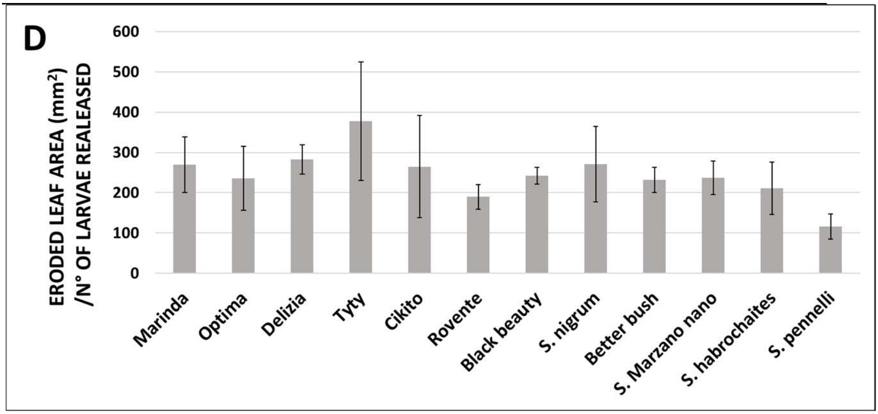
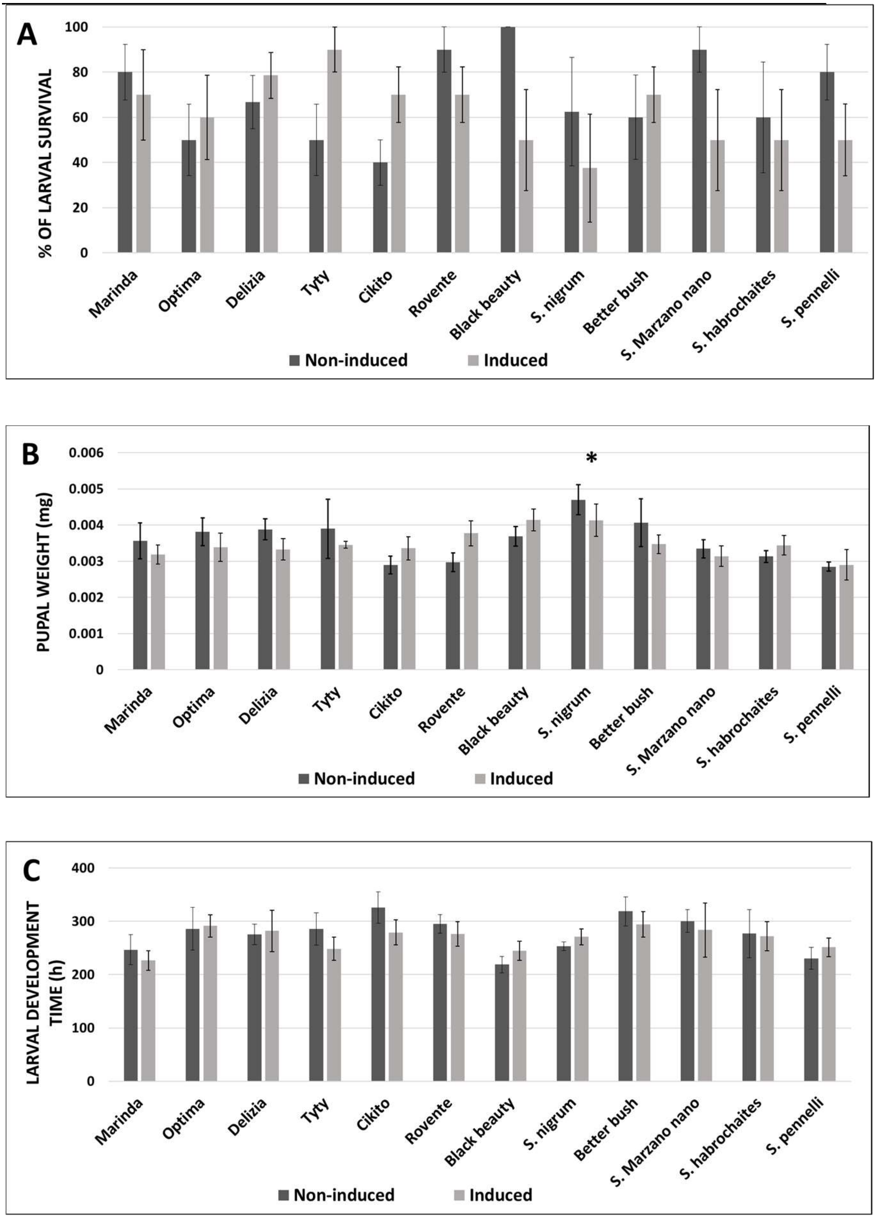


Figure 2.1 *– Mean (±SE) values of (A) the % of larval survival, (B) the pupal weight (mg), (C) the larval development time (h) and (D) the eroded leaf area (mm^2)/n° of* T. absoluta *larvae released on non-induced* Solanum *species / varieties*

2.3.2 Pest-induced defense mechanisms – Secondary metabolites and enzymatic activity

The results of pest-induced defense mechanisms on *T. absoluta* larval survival (Fig. 2.2) show a different effect in each species / variety between the induced and non-induced. There are differences but not significantly when the larvae fed on the eggplant 'Black beauty' and in the commercial tomato varieties 'Cikito', 'San Marzano nano' and 'Tyty'.

For the pupal weight, only for *S. nigrum* ($F_{1,\ 5}$ =22.960; *p*=0.017) showed significant difference between the induced and non-induced plant. Finally, for the larval development time and the eroded leaf area (mm^2)/n° of *T. absoluta* larvae released the *p values* were all >0.05.


A
% OF LARVAL SURVIVAL
0
20
40
60
80
100
Marinda
Optima
Delizia
Tyty
Cikito
Rovente
Black beauty
S. nigrum
Better bush
S. Marzano nano
S. habrochaites
S. pennelli
Non-induced
Induced
B
PUPAL WEIGHT (mg)
0
0.001
0.002
0.003
0.004
0.005
0.006
*
Marinda
Optima
Delizia
Tyty
Cikito
Rovente
Black beauty
S. nigrum
Better bush
S. Marzano nano
S. habrochaites
S. pennelli
Non-induced
Induced
C
LARVAL DEVELOPMENT TIME (h)
0
100
200
300
400
Marinda
Optima
Delizia
Tyty
Cikito
Rovente
Black beauty
S. nigrum
Better bush
S. Marzano nano
S. habrochaites
S. pennelli
Non-induced
Induced

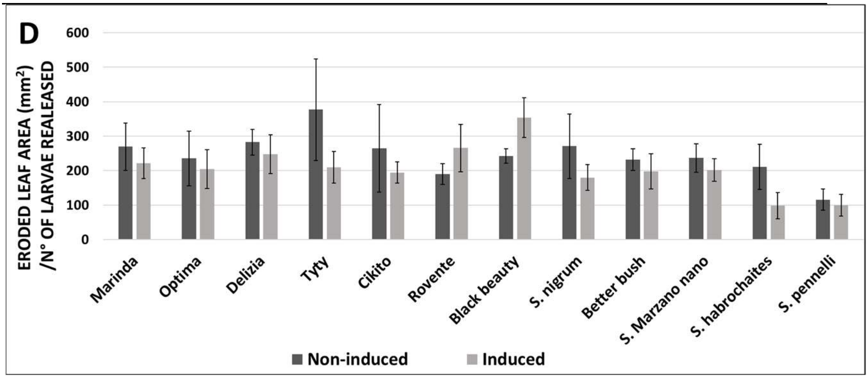


Figure 2.2 *- Mean (±SE) values of (A) the % of larval survival, (B) the pupal weight (mg), (C) the larval development time (h) and (D) the eroded leaf area (mm²)/n° of* T. absoluta *larvae released on either non-induced or induced* Solanum *species / varieties. Asterisks indicate significant differences between treatments within the same species/variety according to the ANOVA at p≤0.05 level*

The results of the analysis of the secondary metabolites show differences between the induced and not induced *Solanum* species / varieties. In Figure 2.3, the comparison of all the chromatograms of the secondary metabolites detected by UPLC-MS is shown. It is possible to notice the differences between the induced and non-induced species in terms of peak area. For example, in the range of retention time between 20 and 30 minutes, it is evident the difference between the peak area of the species / variety that has red-purple color with a bigger area (i.e., for the wild species), compared with that one that has green color with a smaller area (i.e., for the commercial varieties).

In the heatmap, the increasing red color show the intensity of the Mass Spectrometry for each compound (Fig. 2.4). The intense red as the greater quantity of the secondary metabolites and the light red as the lesser quantity. Comparing each induced and non-induced species / variety it could be possible to highlight which one elicited a higher or a smaller quantity of secondary metabolites after the induction by larvae of *T. absoluta*. For example, for the species *S. habrochaites* (green column) in the non-induced part (on the left) the intensity of red color is low, instead of the induced part (on the right) the intensity in higher.

Moreover, the results of a principal component analysis (PCA) show a separation with an overlap of all the replicates of each induced and non-induced species / varieties in two parts (Fig. 2.5). The induced and the non-induced species / varieties are clearly separated in the two overlapped part. For example, the replicates of experiment with the var ‘Black beauty’ induced are located on the left part of the plot instead of the non-induced ones that are on the right part of the plot. This to highlight the possible differences between the secondary metabolites induced and non-induced by *T. absoluta* larvae.

Combining all the results analyzed of the UPLC-MS, it was possible to detect 6524 secondary metabolites that were evaluated with a *p value* ≤ 0.05 comparing the induced and non-induced species / varieties. With the software used it is possible to identify the significant differences between induced and non-induced but to understand the exact identification of which species/varieties or metabolite it is necessary to deepen and evaluate individually with other software. Such analyses are still ongoing.

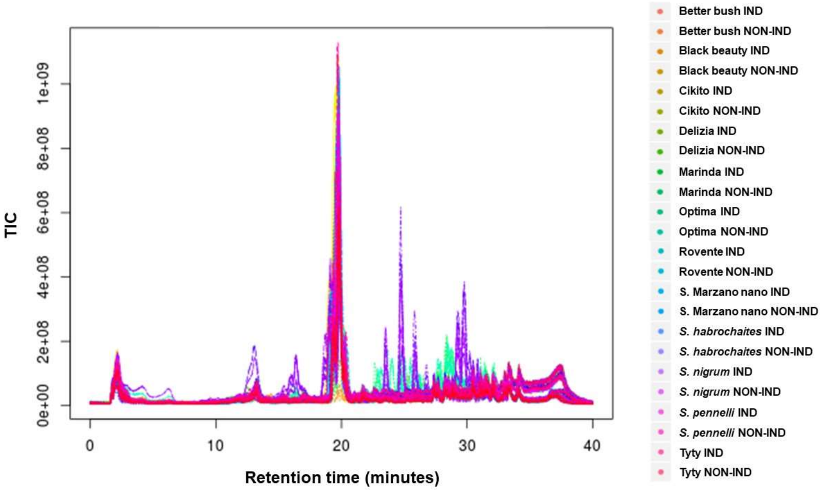


Figure 2.3 *– Comparison of all the chromatograms of secondary metabolic compounds detected by UPLC-MS of* Solanum *species / varieties induced and non-induced by* Tuta absoluta *larvae*

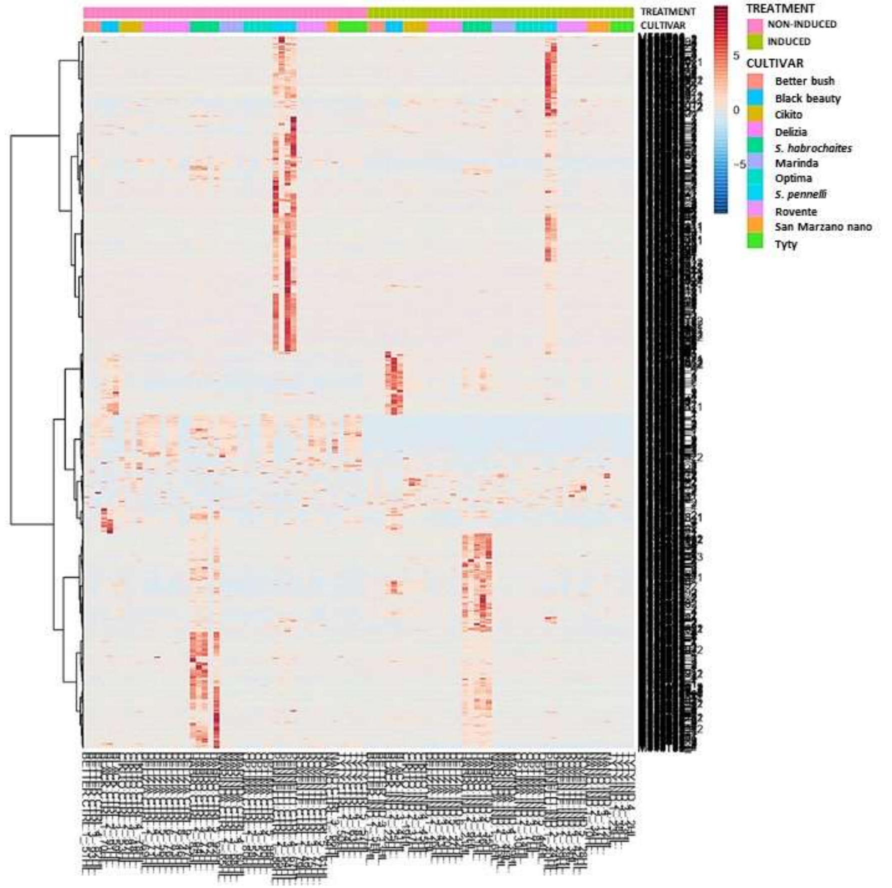


Figure 2.4 *- Heatmap with the secondary metabolic compounds detected by UPLC-MS of* Solanum *species / varieties induced and not induced by* Tuta absoluta *larvae. All the treatments and the* Solanum *species/varieties marked on the top. The intensity of color shows the intensity of the Mass Spectrometry for each compound marked on the edges for all the replicates marked down*

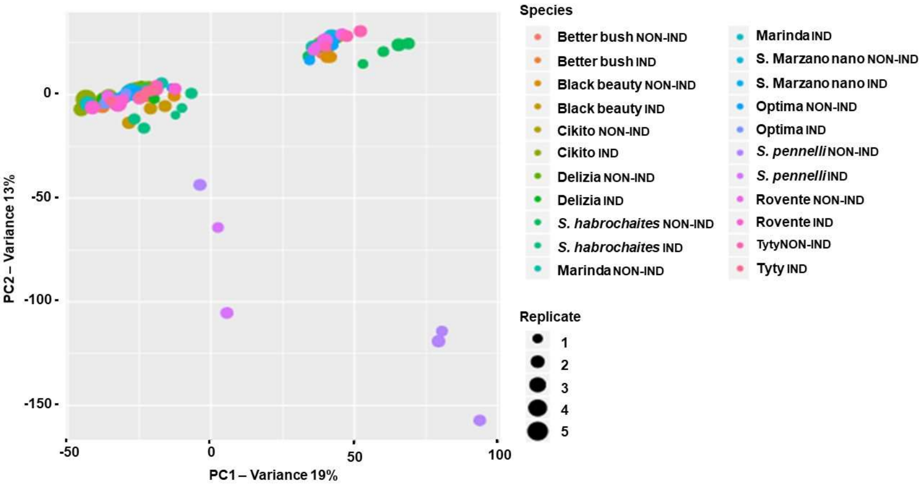
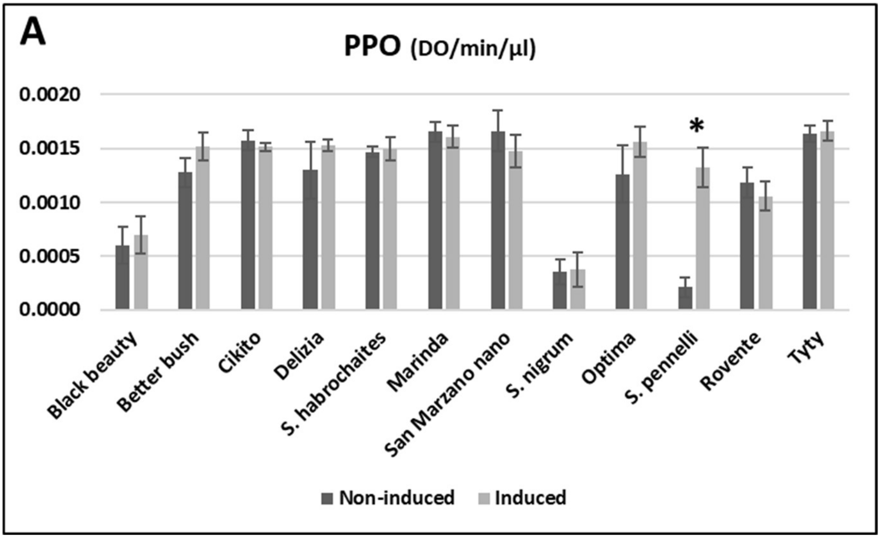


Figure 2.5 *- Score plots of a PCA analysis of secondary metabolic compounds detected by UPLC-MS of* Solanum *species / varieties induced and non-induced by* Tuta absoluta *larvae*

The results of the enzymatic activity, PPO and POD, show that only on *S. pennelli* (PPO: t_9 =0.005, p<0.05; POD: t_9 = 0.016, p<0.05) the analysis highlights a significant difference between the induced and non-induced species / varieties (Fig. 2.6).



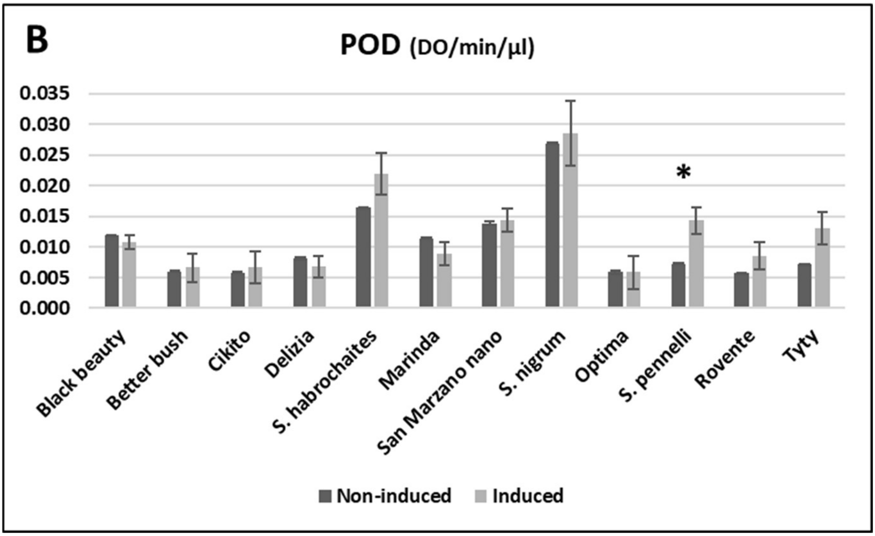


Figure 2.6 *- Results of the investigated enzymatic activities in* Solanum *species / varieties that have been induced or not by* Tuta absoluta *larvae. Mean values of (A) PPO (Polyphenol oxidase) and (B) POD (Peroxidase). Asterisks indicate significant differences between treatments within the same species according to the Student's t-test at $p \leq 0.05$ level*

2.3.3 Discussion

The identification of new biological control tool using the defense mechanisms of wild and commercial *Solanum* species / varieties against *T. absoluta* is one of the purposes of this experimental activity. Several *Solanum* species / varieties were evaluated to identify the different answer to this key pest in terms of plant suitability bioassays, induced secondary metabolites and enzymatic activity. The results show the potential effects against *T. absoluta* of the metabolites produced in the induced plants, especially in the eggplant variety 'Black beauty' and in the tomato varieties 'San Marzano nano' and 'Cikito' as showed by pest-induced defense mechanisms bioassay. The results suggest that the feeding activity by larvae of *T. absoluta* increases or decreases the production of plant secondary metabolites differently in each species / variety. This could be due to the different metabolomic approach of each species / variety to the insect attack. For example, the saliva of the insect contains proteins with different functions that alters the response and the interaction with each plant (Will et al. 2009; Hogenhout & Bos 2011). The metabolic reaction and the activation of different secondary metabolites could be different in each species / variety depending on the saliva effect.

Moreover, for the different metabolic responses of each species / variety depends on the differences in insect development. This could be due for the potential direct antibiosis and antifeedant activity of induced substances on *T. absoluta* behavior and development (Weinblum et al. 2021). The accumulation of induced defense metabolites, such as glycoalkaloids, bioactive natural products used by plants as chemical defenses providing good resistance traits against insect (Abdelkareem et al. 2017). For example, tomatine is a glycoalkaloid compound known to be toxic for insect. Generally, after the attack of insects, the tomatine production increases reducing the performance and the survival of the insect (Larbat et al. 2016).

About the results of the enzymatic activity, a wild species S. *pennelli* show a significant difference between induced and non-induced *Solanum* species / varieties. This result could be derived probably from the exposure time of the species / varieties to larvae of *T. absoluta*. It was evaluated the enzymatic activity only three days after the larvae inoculation, more days of exposure maybe could enhance the activation of the enzymes in the other species / varieties. Moreover, this result could be due to the different reaction of each species / variety to the pH of the saliva of the larvae of *T. absoluta*. It was studied how the pH of the saliva, if it is basic or acid, could alterate the oxidative enzyme activity and the subsequent chemical reactions (Duffey & Stout 1996).

These results encourage the use of resistance plant ability to protect themselves against insects. Different defense mechanisms are involved in this answer not only metabolites and enzymatic activity, for these reasons further studies need to be performed to have completed results. For example, to test the identified secondary metabolites of induced *Solanum* species / varieties as possible bio-insecticides and at the same time evaluate the sub-lethal effects on natural enemies in the tri-trophic levels.

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3 Chapter 3: Tri-trophic interactions through plant volatile induction and expression of defense genes elicited by *Tuta absoluta* larvae in *Solanum* spp.

Simona Tortorici et al., in preparation for submission to *Journal of Chemical Ecology*

Tri-trophic interactions through plant volatile induction and expression of defense genes elicited by *Tuta absoluta* larvae in *Solanum* spp.

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3.1 Introduction

Volatiles Organic Compounds (VOCs) related to herbivory are called Herbivore-Induced Plant Volatiles (HIPVs) and are emitted from plant leaves after herbivore damage. These volatile compounds have been shown to attract natural enemies and locate their host or prey, or to repel insect herbivores, or they are implicated in inter- and intra-plant communication (Ayelo et al. 2021c; Pirk 2021).

Following insect attack, plants usually respond by releasing a blend of volatiles that is strongly different from those of undamaged plants and that can elicit direct anti-herbivore defenses and indirect defense mechanisms, e.g. through the attraction of natural enemies (Kost & Heil 2006; Sun et al. 2021). These mechanisms work as natural regulators of pest populations and understanding how these interactions work can lead their exploitation in agricultural ecosystems, in order to reduce both pest populations and the use of chemical insecticides (Kant et al. 2015; Kersch-Becker & Thaler 2019).

In this framework, many studies focused on *Solanum* species that could be considered as a laboratory model for plant defense mechanisms involved in answer to pests and for the attraction of natural enemies (Gontijo et al. 2019; Conboy et al. 2020; Ayelo et al. 2021c; Chen et al. 2021). Indeed, in this study, pest induced defense mechanisms in various *Solanum* species and tomato varieties were assessed at various levels. The *Solanum* species and tomato varieties were exposed to larvae of the key tomato pest *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) and the effect of the emitted VOCs and HIPVs on adults of *T. absoluta* and *E. formosa* were evaluated by observing the insect behavior in a dual choice test in Y-tube olfactometer. Moreover, the expression of two *Solanum* defense genes, TPS 20 and PR I-II, involved in the expression of β-phellandrene and salicylic acid respectively, was performed.

The results of this study aim to evaluate the possibility of developing new biocontrol systems and strategies to manage pests in agriculture, through a better understanding on how tri-trophic interactions among plant, insect pests and natural enemies are influenced by each other.

3.2 *Materials and methods*

3.2.1 *Plant rearing*

Solanum lycopersicum var 'San Marzano nano' was used as host tomato plant to rear *T. absoluta*; while plants for the experiments were the following: *S. lycopersicum* var 'Better bush' ® (Tomato Growers, USA), var 'San Marzano nano'® (Bavicchi, Italy), var 'Optima'® (Dotto, Italy) as experimental models (Peterson et al. 1991; Catola et al. 2018; Alfosea-Simón et al. 2020). *S. lycopersicum* var 'Cikito'® (Monsanto, USA), var 'Delizia'® (Clause, France), var 'Marinda'® (Nunhems, Germany), var 'Rovente'® (Monsanto, USA) and var 'Tyty'® (Syngenta, China) as commercial tomato varieties typical of the Mediterranean protected tomato systems. Moreover, *S. nigrum* seeds were used for the experiment, and were collected near the Di3A in Catania in July 2017. *Solanum habrochaites* (CRB-Leg code: T300007) and *S. pennelli* (CRB-Leg code: T300107) were originated from the seed collection of the research unit of Génétique et Amelioration des Fruits et Légumes (UR1052) of the Institut national de recherche pour l'agriculture, l'alimentation et l'environnement (INRAe, France). Finally, plants of *S. melongena* var 'Black beauty'® (Dotto, Italy) was used for the experiments.

Solanum species / varieties seedlings were grown initially in a climatic chamber at 25°C, 60±10% R.H. and 16L:8D photoperiod, and after they reached 10 cm in height, they were moved in the greenhouse at 24°C, 60±10% R.H. and natural daylength. Experiments were carried out with plants with three fully expanded true leaves.

The plants for the experiments had been pre-infested by releasing eight second instar larvae of *T. absoluta* on each second and third leaf. For the non-induced (control) plants a simulation of the inoculation of the larvae on each third and fourth leaf was done to nick the trichomes with a brush.

After three days of pre-infestation, each tested plant was ready for the identification of the volatile compounds with GC-MS, for the olfactometer bioassays, or to undergo to the analysis of the expression of two genes involved in the defense mechanisms.

3.2.2 Insect colonies

Adults and larvae of *T. absoluta* used for the experiment were collected from the laboratory insect rearing of the Agriculture, Food and Environment Department (Di3A) of the University of Catania (IT). The colony was originated from individuals collected in many commercial tomato crops of South Eastern Sicily (Catania, Ragusa, Siracusa) and it was established in 2008. It was located in a chamber with controlled environmental conditions, as 25 ± 2 °C of temperature, 50 ± 10 % of relative humidity and L14:D10 of photoperiod. The individuals of *T. absoluta* were reared on *S. lycopersicum* var 'San Marzano nano' as described in Zappalà et al. (2012). Adults of the parasitoid *E. formosa* used for the experiments were supplied by *Koppert biological system*.

3.2.3 Volatile analysis

VOCs and HIPVs emitted by leaves of tested plants were collected via solid-phase microextraction (SPME; Supelco) using grey fibers following the experimental protocol described by Verzera et al (2011). Plants were put in hermetic glass bottles to be sampled. Three replicates for each induced and non-induced variety were carried out. The SPME fiber was inserted through a small opening in the cover for 30 min of exposure and after it was analyzed by GC-MS. A Varian 3800 gas chromatograph directly interfaced with a Varian 2000 iontrap mass spectrometer (GC-MS) (Varian Spa, Milan, Italy) was used under the following conditions: injector temperature 260 °C; injection mode, splitless; capillary column, CP-Wax 52 CB, 60 m, 0.25 mm i.d., 0.25 lm film thickness (Varian Chrompack Italia s.r.l., Milan, Italy); oven temperature, 40 °C with an increase up to 200 °C at rate of 3 °C/min and to 240 °C at 10 °C/min; carrier gas, helium at constant pressure of 10 psi; transfer line temperature, 250 °C; acquisition range, 40–200 m/z; scan rate, 1 scan s-1. Data were acquired in total ion current (TIC) mode.

3.2.4 Y-tube olfactory bioassays

In order to perform the olfactory bioassays, the following plant species / varieties *S. lycopersicum* var 'Cikito', var 'Delizia', var 'Marinda', var 'Optima', var 'Rovente', var 'San Marzano nano', var 'Tyty' and *S. melongena* var 'Black beauty' were tested. The plant species / varieties to be tested in the olfactory bioassays were chosen after the analysis of the volatile amounts produced by induced and non-induced species, selecting those that had a more intense induced volatile production (see the results in the *Volatile analysis* section).

A Y-tube olfactometer was used. It consisted of a Y-shaped glass tube of 1.5 cm diameter, with an entry arm 18.5 cm long and two side arms of 18.5 cm in length and 70° angle (Naselli et al. 2017). The horizontal orientation of the Y-tube was used for both targets *T. absoluta* and *E. formosa*. An air pump was connected to the Y-tube regulated at 100 mL/min. The Y-tube was illuminated by a lamp of 22 W cool-white fluorescent circular tubes 80 cm above the arms.

A new emerged, starved and mated *T. absoluta* female, was tested in the choice test comparing two odor sources: induced plant and non-induced plant of the same varieties. The pest was released at the entrance of the horizontally oriented olfactometer, and a choice was considered when the insect reached the distal part of one arm of the olfactometer. Each female was evaluated for maximum 10 minutes.

At the end of the behavioral observation *T. absoluta* adults were killed in ethanol and their distal part of the abdomen observed under the binocular to confirm the sexual identification. Thirty-two (32) females were tested per each *Solanum* species / variety. Females that did not make a choice and those wrongly identified as females were discarded from the dataset (Biondi et al. 2015).

The Y-tube used for *E. formosa* bioassays was 1.5 cm of diameter and 6 cm for the entry and the side arms. The parasitoid was released at the entrance of the horizontally oriented olfactometer, and a choice was considered when the distal part of one arm of the olfactometer was reached and when the parasitoid stayed there for at least 1 minute more than the time needed to perform the choice. The maximum time per each female choice was 10 minutes. Thirty-two (32) females were tested per each *Solanum* species / variety. Females that did not make a choice were discarded from the dataset (Ayelo et al. 2021c).

For both insect species, the olfactometer was turned every four tested females to eliminate the odor source bias. Every eight tested females the plant was changed. The olfactometer was rinsed up with ethanol (90%) after every tested odor source. At the beginning of the experiment and every time the plant was changed, the plant was left inside the glass jars fifteen minutes to let the volatiles odor circulate in the system. The bioassays were conducted between 09.00 and 18.00, at 24±1°C and 60±10 % RH.

3.2.5 *Gene expression*

The analysis of the expression of two defense genes was only for three tomato varieties (i.e., ‘Optima’, ‘Rovente’ and ‘Marinda’) that showed significant results in the insect olfactory responses (see the results in the *Y-tube olfactory bioassays* section). The expression of the TPS20 gene, which is responsible for the expression of β-phellandrene and belongs to the Terpene Synthase group (Falara et al. 2011), and PR I-II gene (pathogenesis-related gene), which is responsible for the expression of salicylic acid, was performed by reverse transcription of the total RNA isolated from plant tissue after induction of defense mechanisms. These two genes and their related metabolites have been showed to be related to induced defenses in tomato (Gu et al. 2000; Herman et al. 2007; Coppola et al. 2018). Both genes were compared to the constitutive gene EF1 (Elongation Factor 1) as described by Pérez-Hedo et al. (2015).

Vegetal tissues for the gene expression were taken and prepared only from the induced and non-induced varieties ‘Rovente’, ‘Marinda’ and ‘Optima’ with three replicates for treatment. The apical part of each plant was labelled and stored in climatic chamber at -80±2 °C. For RNA extraction, the samples were grinded in mortar with a pestle using liquid nitrogen to keep low temperature in order to avoid metabolic alteration of the samples, and 30 mg of crushed plant tissue was collected in a labelled Eppendorf and stored in climatic chamber at -80±2 °C.

Total RNA was extracted from the harvested tissues using SV Total RNA Isolation System kit (Promega Corp., Madison, WI, USA) following manufacturer's protocol (available at: www.promega.com/protocols/). After extraction, the RNA was treated with RNase-free DNase (Promega Corp., Madison, WI, USA) to eliminate potential DNA contamination.

After RNA extraction and purification, the next step was the reverse transcription (RT) of RNA in complementary DNA (c-DNA), performed using the Reverse Transcription System kit (Promega Corp., Madison, WI, USA) following the protocol procedures. RT reactions were composed by 4 µL of 25mM MgCl2, 2 µL of RT 10x Buffer, 2 µL of dNTP Mixture 10 mM, 0.5 µL of Recombinant RNasin Ribonuclease Inhibitor, 15 uL of AMV Reverse Transcriptase, 0.5 µg of Random Primers, 1 µg of RNA and ultrapure water until up to 20 µL in volume.

The samples were processed in the thermal cycler with three temperature steps: 42 °C for 15 min, 95 °C for 5 min, and 0-5 °C for

5 min, at the end the samples were stored at -20±2 °C.

cDNA was amplified by a real-time quantitative PCR (RT-qPCR) that was performed using a PCR amplification kit. To perform RT-qPCR we used 10 µL of 2x SensiFAST SYBR®& Fluorescein Mix, 0.8 µL of 10µM Forward Primer, 0.8 µL of 10µM Reverse Primer, 1 µL of c-DNA and ultrapure water until up to 20 µL in volume. Samples were amplified with RT-qPCR thermal cycler (Bio Rad iCycler iQ Multicolor Real-Time PCR Detection System) with one cycle of 95°C for 2 minutes, 40 cycles at 95°C for 5 minutes, 60°C for 20 seconds and 72°C for 10 seconds, followed by a final stretching step of 95°C for 2 minutes.

Three technical replicates were performed for each biological replication. The gene expression of the TPS20 and of the PR I-II genes was obtained using the 2ΔCt formula. The expression of EF1 was used as a standard control gene for normalization.

3.2.6 Data analyses

The differences in the amount of each volatile compounds of the induced and non-induced *Solanum* species / varieties were performed with the online platform Xcms^{tm}. The non-parametric test Kruskal-Wallis comparing all the chromatograms was performed with $p \leq 0.05$. The X^2 test was used to test the hypothesis that the distribution of the choices of *T. absoluta* and *E. formosa* females tested in olfactory bioassay between the two odor sources deviated from a null model in which the odor sources were chosen with equal frequency. The results were analyzed with the software Microsoft Excel and were expressed as percentage of preference.

The differences in the expression of TPS20 and PR I-II genes and the constitutive EF1 gene in induced and non-induced *Solanum* varieties were analyzed with one-way analysis of variance (ANOVA) and the means were then compared by Least Significant Difference (LSD) test ($p \leq 0.05$). Statistical analysis was conducted with the Software R

(Version 4.1.1 (2021-08-10), package “agricolae” version 1.3-5).

3.3 Results

3.3.1 Volatile analysis

The results of the analysis on the collected volatile data show a significant difference between the induced and non-induced *Solanum* species / varieties. In Figure 3.1, the comparison of all the chromatograms of the volatile compounds detected by GC-MS is shown. It is possible to notice the differences between the induced and non-induced species in terms of peak area. For example, in the range of retention time between 10 and 20 minutes, it is evident the difference between the peak area of the species / varieties that has purple color with a smaller area (for example the species ‘*S. pennelli’*), compared with that one that has light blue color with a bigger area (for example the variety ‘Rovente’).

In the heatmap, as the name itself indicates, the intensity of red color shows the increasing quantity of the Mass Spectrometry for each compound (Fig. 3.2). The intense red as the greater quantity of the volatile compound and the light red as the lesser quantity. Comparing each induced and non-induced species / variety it could be possible to highlight which one elicited a higher or a smaller quantity of volatile compounds after the induction by larvae of *T. absoluta*. For example, for the var ‘San Marzano nano’ (light orange column) in the non-induced part (on the left) the intensity of red color is very low, instead of the induced part (on the right) the intensity in clearly higher.

Moreover, the results of a principal component analysis (PCA) show a separation with a slight overlap of some replicates of each induced and non-induced species (Fig. 3.3). For example, the wild species with color tending to purple are overlapped on the right part of the plot instead of the commercial varieties with color tending to orange and green are in the middle. This to highlight the possible differences between the volatiles emitted by species and varieties and the difference attractivity of *T. absoluta* and *E. formosa*.

After the analysis of all the data, the ratio of the chromatogram retention time to the relative retention time correspond to a tolerance of ± 0,5 % (Fig. 3.4). This is an important result for the identification of the compounds in GC-MS. Because if retention times are slightly longer than ± 0.5 % as expected range of retention time, a peak might not be detected correctly.

Combining all the results analyzed of the GC-MS, it was possible to detect 78 volatile compounds comparing the differences between the induced and non-induced within each species / variety. With the software used it is possible to identify the significant differences between induced and non-induced but to understand the exact identification of which species / varieties or compounds it is necessary to deepen and evaluate individually with other software. Such analyses are currently ongoing.

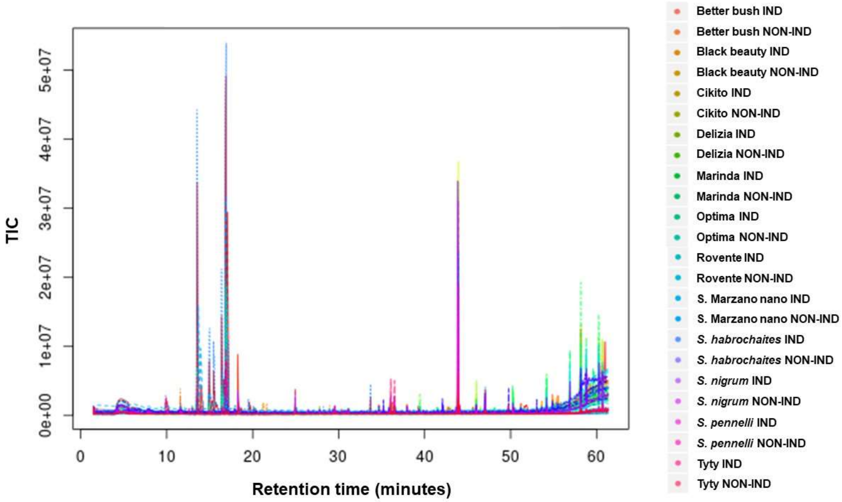

Figure 3.1 *– Comparison of all the chromatograms of volatile compounds detected by GC-MS of* Solanum *species / varieties induced and non-induced by* Tuta absoluta *larvae*

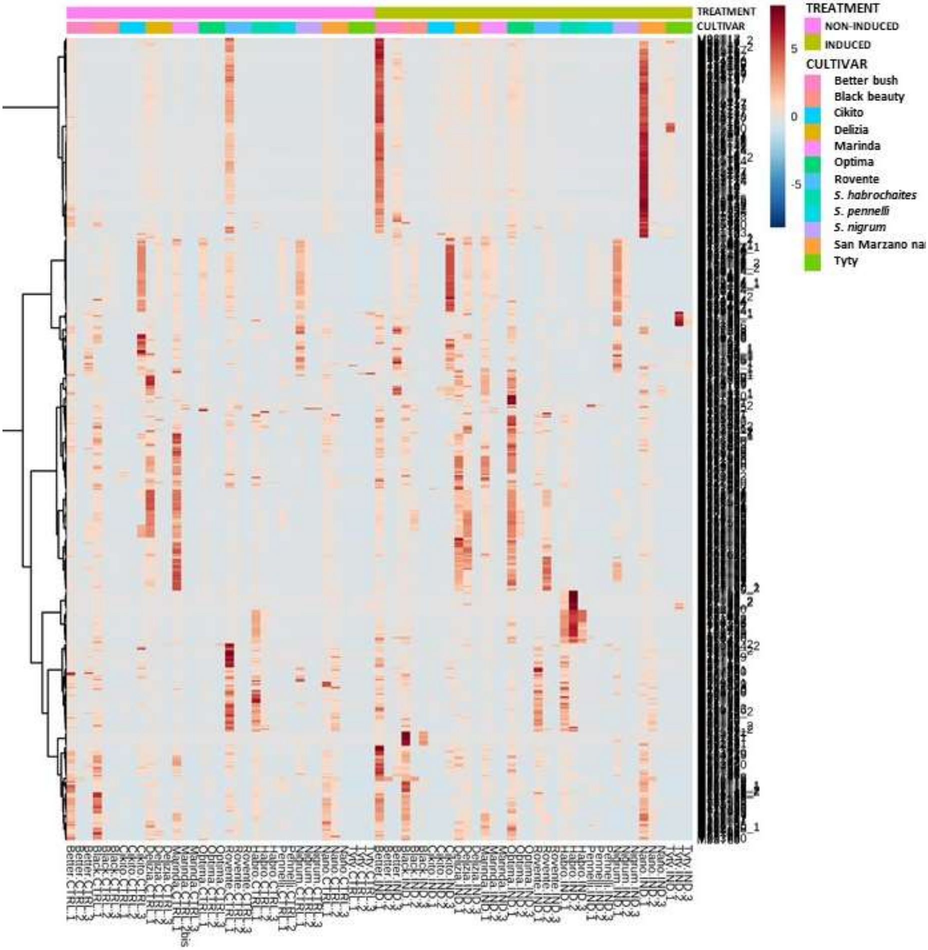

Figure 3.2 *- Heatmap with the volatile compounds detected by GC- MS of* Solanum *species / varieties induced and non-induced by* Tuta absoluta *larvae. The intensity of color shows the intensity of the Mass Spectrometry for each compound marked on the edges for all the replicates reported on the x axis*

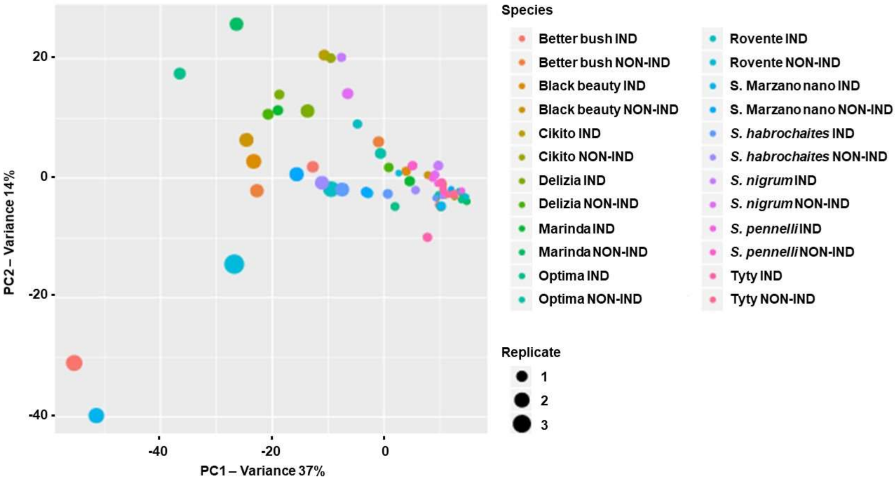

Figure 3.3 *- Score plots of a PCA analysis of volatile compounds detected by GC-MS of* Solanum *species / varieties induced and non-induced by* Tuta absoluta *larvae*

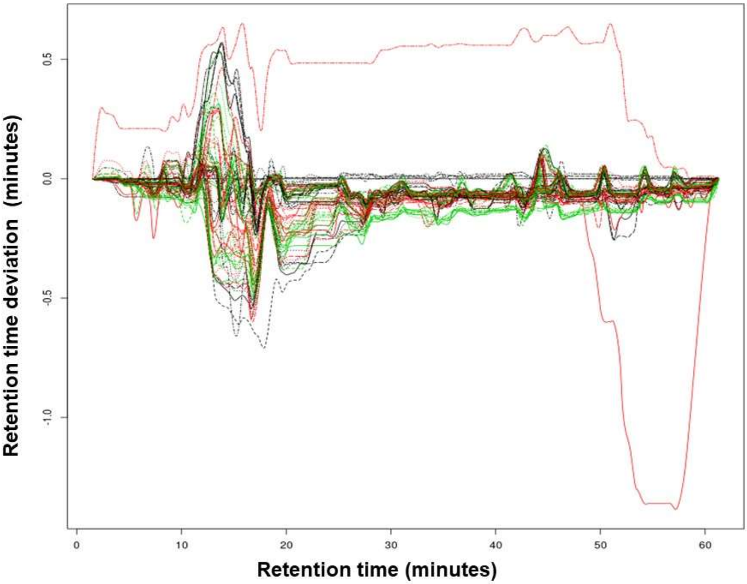

Figure 3.4 *- Ratio of the retention time of the chromatograms of all volatile*

compounds detected by GC- MS of Solanum *species / varieties induced and non-induced by* Tuta absoluta *larvae, to the relative retention time corresponding to a tolerance of ± 0,5 %*

3.3.2 Y-tube olfactory bioassays

In most of the dual choice tests, *T. absoluta* adults were more attracted by the volatiles emitted by induced plants rather than healthy ones. However, such a trend was significant only for two varieties 'Marinda' (X^2= 6.081; p= 0.013) and 'Optima' (X^2= 3.812; p= 0.050) (Fig. 3.5) and for all the other species / varieties the *p value* was >0.05. For the variety 'Cikito' and 'San Marzano nano' the non-induced plants were preferred but not significantly different compared to the induced ones.

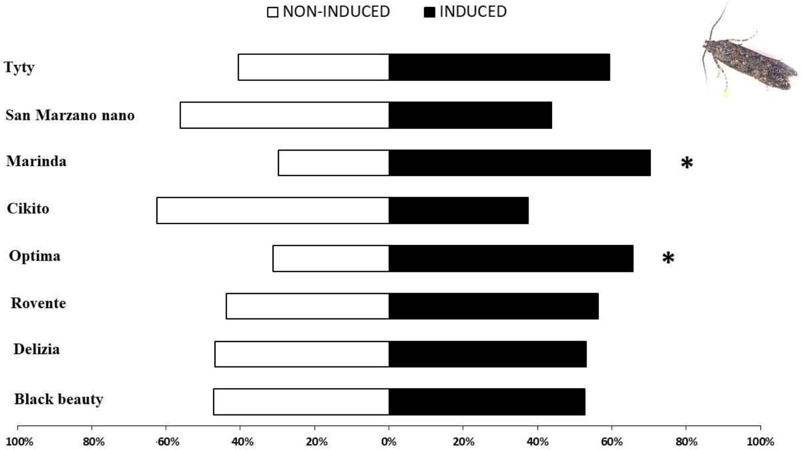

Figure 3.5 - *Olfactory response of* Tuta absoluta*: percentage of females showing a response toward the volatile cues produced by induced and non-induced* Solanum *species / varieties. Asterisks indicate statistical significance of the* X^2 *tests at confidence interval level of 95%*

The olfactory bioassays with *E. formosa* gave significant results only in the case of the test with the variety 'Rovente' (X^2= 4.500; p= 0.033) (Fig. 4.6). Overall, the results showed a higher attraction toward *E.*

formosa by the volatiles emitted by induced plants rather than non-induced ones. Indeed, for all the other species / varieties the *p value* was >0.05. 'San Marzano nano' and 'Optima' are the two varieties in which the non-induced plant is the most preferred although not significantly.

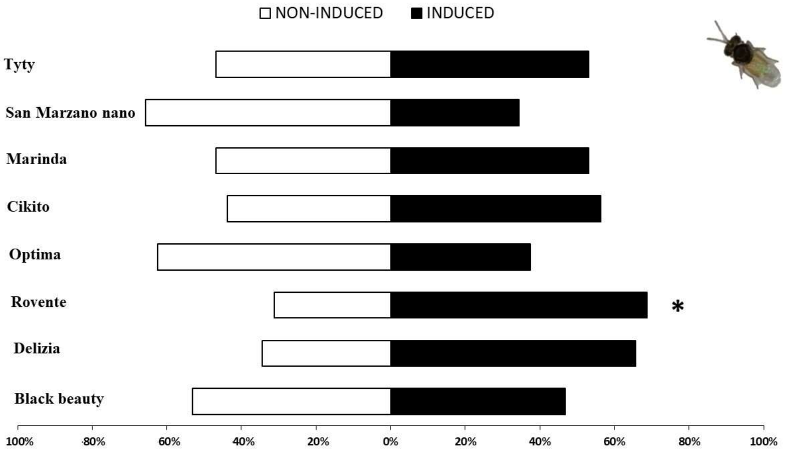

Figure 3.6 *- Olfactory response of* Encarsia formosa*: percentage of females showing a response toward the volatile cues produced by induced and non-induced* Solanum *species / varieties. Asterisks indicate statistical significance of the* X^2 *tests at confidence interval level of 95%*

3.3.3 Gene expression

The results of gene expression analysis of the apical part of both induced and non-induced plants confirm that induced plants have β-phellandrene and salicylic acid expression, respectively codified by genes TPS20 and PR I-II. These results show a significant difference between induced and non-induced plants in terms of expression of PR I-II after the attack of *T. absoluta* larvae in 'Optima' ($F_{1, 6}$=32.370; *p*=<0.001), 'Rovente' ($F_{1, 6}$=25.360; *p*=<0.001) and 'Marinda' ($F_{1, 6}$=27.700; *p*=<0.001) (Fig. 3.7). While no significant difference, between induced and non-induced plants, in terms of expression of TPS20 after the attack of *T. absoluta* larvae was observed in neither of the three varieties tested (‘Optima’: $F_{1,\ 6}$ =0.043; p=0.839; ‘Rovente’: $F_{1,\ 6}$ =1.221; p=0.285; and ‘Marinda’: $F_{1,\ 6}$ =0.504; p=0.488).

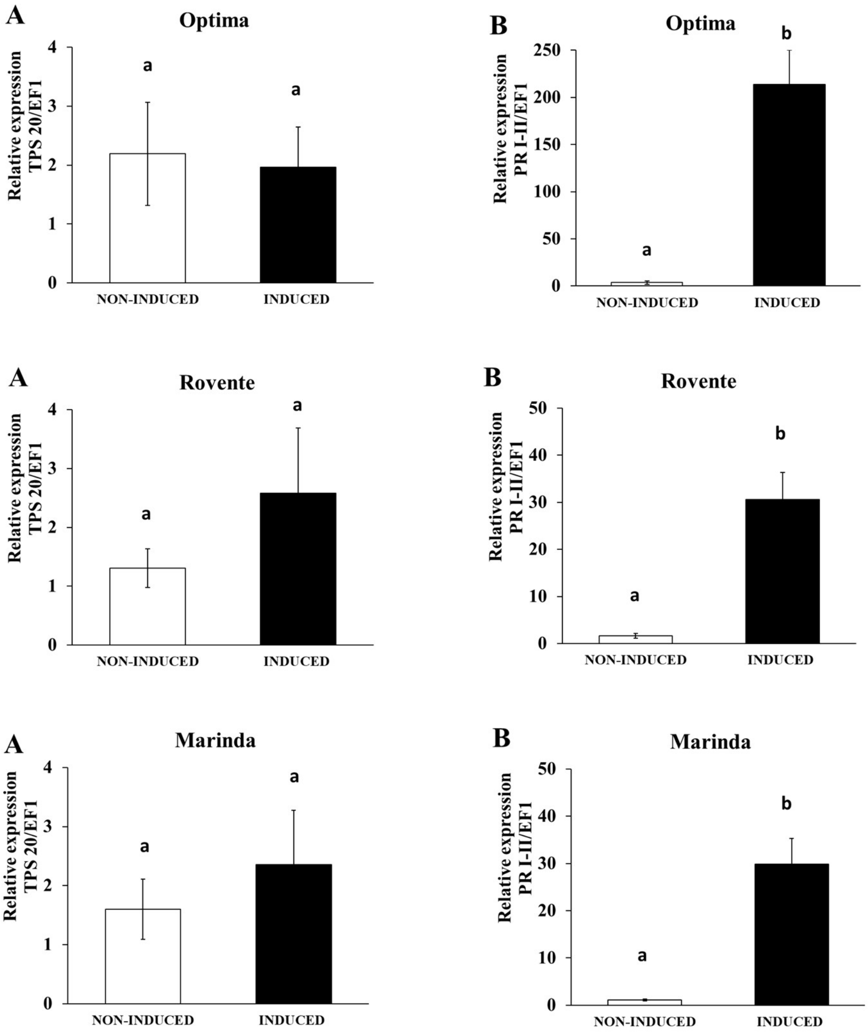

Figure 3.7 *- Expression analysis of TPS20 (A), PR I-II (B) of the apical part of* Solanum *varieties induced and non-induced by* Tuta absoluta *larvae. Transcription levels were normalized to the expression of EF1 measured in the same sample. Data are presented as the mean of three independent analyses of transcript expression relative to a housekeeping gene ± SE (n = 3). Bars bearing different letters are significantly different based on ANOVA, LSD test ($p < 0.05$)*

3.4 Discussion

Pest induced tomato plant defense mechanisms can interfere with the attraction of pests and natural enemies. For this reason, it is interesting to investigate how to use these mechanisms in a IPM context to increase the sustainable approach to control key pest of cultivated crop. In this frameowork, it was investigated the production of VOCs and HIPVs induced by *T. absoluta* larvae in *Solanum* varieties / species and the olfactory response of adults of *T. absoluta* and *E. formosa*. Furthermore, we investigated the relation between *T. absoluta* larvae induction and the expression of two defense genes in *Solanum* plants. The results show that some species / varieties can be more attractive for both *T. absoluta* and *E. formosa* adults after being induced by *T. absoluta* larvae. This could be show by the difference between all the chromatograms of GC-MS results and in the heatmap of the species / varieties induced or non-induced by *T. absoluta* larvae Moreover, all the volatiles were matched and 78 volatile compounds were evaluated with a *p value* ≤ 0.05. These results are only based on the Mass Spectrometry of each peak of the volatile compounds, but at this step it is necessary to identify each compound and to compare it with literature to know how they interfere with plants, pests and natural enemies (Subramani et al. 2021).

The bioassays conducted in olfactometer showed that preferences of both insects between induced and non-induced plants changed according to the variety. The varieties 'Marinda' and 'Optima' showed to be significantly more attractive to *T. absoluta* after induction probably because the volatiles emitted by the already damaged plant are silencing by the insect salivary enzyme that closes plant stomata and inhibits volatile release (Lin et al. 2021). While *E. formosa* showed to be significantly more attracted by the variety 'Rovente' after induction. This result confirms that the induction by larvae stimulated the induced volatile emission because generally, a natural enemy is attracted by a plant with the presence of the prey and induced volatiles instead of that one without prey and healthy plant (Naselli et al. 2017). These findings led us to a gene expression analysis to evaluate defense-related genes TPS20 and PR I-II expression. Indeed, results showed a high defense response in the induced plants, resulting in an increase of production of one of the two defense metabolites which is salicylic acid, related to PR I-II genes expression (Caparros Megido et al. 2014; Shi et al. 2016; De Backer et al. 2017).

Similarly, the parasitoid *E. formosa* was more attracted by plants induced by *T. absoluta* larvae, supporting the hypothesis that adults of *E. formosa* are attracted by the presence of a key pest rather than by healthy tomato plants (Gelman et al. 2005). Indeed in 'Rovente', in which the attraction of *E. formosa* adults was significantly different between induced and non-induced plants, the production of β-phellandrene was higher in induced plant than in non-induced, this to highlight the role of β-phellandrene as defense volatile compound implicated in tri-trophic interaction. Conversely, in 'Optima', in which the attraction of *E. formosa* adults was not different between induced and non-induced plant, the production of β-phellandrene was not significantly different neither.

Future perspective could be represented by potential field applications of *Solanum* HIPVs with the aim to reduce pest populations both directly, disrupting pest attraction towards the crop and indirectly attracting natural enemies. Indeed, it could be interesting to select some VOCs or HIPVs useful as attractant or repellent compounds in greenhouses. Several studies show that HIPVs are important signals for generalist parasitoids or predators in locating their prey, thus HIPVs could be used in biocontrol strategies by attracting natural enemies and decreasing pest population (De Backer et al. 2015; Silva et al. 2018; Ayelo et al. 2021a, 2021b; Chen et al. 2021; Pérez-Hedo et al. 2021).

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4 General discussions and conclusions

This thesis contributes to provide further insights into the evaluation of new sustainable and integrated control tools for optimizing the natural plant defenses, as described in Chapter 1, and providing new techniques against key pests of *Salanum* species / varieties such as *T. absoluta*. In this framework, our results demonstrated that the defense mechanisms of *Solanum* species / varieties could be a possible way to sustainable pest control. It was shown that the production of defense compounds is influenced by the attack of herbivores. More in details, in Chapter 2, after a previously analysis, the investigated *Solanum* species / varieties proved to have a higher production of secondary metabolites, probably also in terms of glycoalkaloids such as the tomatine, after the attack of *T. absoluta* larvae. Indeed, the behavior parameters of *T. absoluta* larvae investigated in the bioassays show a difference between the induced and non-induced species where the performance of the tomato key pest is reduced. That results could be compared with that ones in Chapter 3, because after induction of *Solanum* species / varieties by larvae of *T. absoluta*, the evaluation of volatile compounds emitted by these plants was overexpressed Moreover, that overexpression could help us to understand the olfactory response of the pest *T. absoluta* and the parasitoid *E. formosa* confirming the significative expression of defense genes analyzed.

In conclusion, it could be said that the attack of the herbivore *T. absoluta* could influence the eco-physiological responses of the plants, and subsequently influencing the pest performance. This contributes to underline the possibility of using these effective induced compounds and volatiles in an integrated and sustainable pest management. Nevertheless, the outcomes of the present work need to be supported by further studies on the evaluation of the bioactive capacity of this possible compound used for example as biopesticide or as attractive and repellent substances to natural enemies and pollinators. Also, it could be interesting to investigate these defense mechanisms in other crops of relevant economic interest. Finally, the results need to be reproduced in field applied perspective to confirm the good efficacy of these new sustainable tools.

5 Appendix A: Other scientific manuscripts

5.1 *Silica-Microencapsulated Orange Oil for Sustainable Pest Control*

Marzia Sciortino, Antonino Scurria, Claudia Lino, Mario Pagliaro, Fabio D’Agostino, Simona Tortorici, Michele Ricupero, Antonio Biondi, Lucia Zappalà and Rosaria Ciriminna

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Silica-Microencapsulated Orange Oil for Sustainable Pest Control

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Mechanically extracted from the orange peel cells via a water jet stream before fruit squeezing for juice production, orange oil is a valued essential oil (EO) mostly composed of d-limonene.[1] Edible and with an exquisite scent, orange oil finds its major applications in the food, beverage, cosmetic, perfumery and oil industries.[1]

Its current value is so high (exceeding \$10 kg^{-1}) that today's orange juice makers earn a large and increasing share of their revenues from its sale.[2] The 15 year (2003–2017) average global supply of orange oil has been around 57 000 tons, with a 9000 tons shortage in 2016/2017 due to constant decline of Florida's production hit by citrus greening disease. The price of orange oil has gone from then record high \$9.45 kg^{-1} in the summer of 2017 for bulk samples,[2] to over \$11.50 kg−1 in the summer of 2019.[3]

The amount of aldehydes contained in orange oil, both terpenes (neral and geranial) and aliphatic aldehydes (decanal and octanal), crucially affects the olfactory notes of orange oil. The concentration of carbonyl compounds, which substantially influences the orange oil value and price, exceeds 1% in EOs from organically grown oranges, whereas is less than 0.4% in oils containing pesticides.[4]

Citrus essential oils are a promising tool for the sustainable control of economically relevant agricultural pests, but their field application is limited by several constraints such as the high instability and degradability patterns. However, the encapsulation of essential oils into nanostructures can overcome such limitations, ensuring the preservation of the insecticidal properties.[5,6]

Among the many and varied microencapsulation technologies, the encapsulation of organic liquids in porous SiO_2 microspheres stands out for multiple benefits, including the vastly enhanced chemical and thermal stability of the microencapsulated organic species, high mechanical strength, ease of handling (since silica does not carry a static charge and is always free flowing), and dispersion of the microspheres in different liquid media.[7] Silica, furthermore, is a biocompatible and environmentally benign (biodegradable) material ideally suited for biological, medical and pharmaceutical applications.[8]

Co-formulated with other substances such as urea and borax, orange oil is highly effective in killing or repelling several noxious insects, fungi, bacteria and even viruses.[9] At least two broad-spectrum biopesticides based on orange oil have been recently commercialized.[9] Orange oil terpenes, however, are easily oxidized when exposed to high temperature, oxygen and humidity, namely, the typical open field conditions.

From complex coacervation with biopolymers[10] through spray-drying with gelatin or lignin[11] several techniques have been described to microencapsulate orange oil. To the best of our knowledge, encapsulation in spherical SiO_2 microcapsules has not been reported. In this study we describe the outcomes of using sol–gel microencapsuled crude orange oil, herein dubbed SiliOrange, formulated in water as insecticide against the cotton aphid *Aphis gossypii* Glover (Hemiptera: Aphididae) and the cotton leafworm *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) under laboratory conditions. Both insect pests are extremely polyphagous and can cause serious economic losses to a multitude of agricultural and ornamental crops.[12,13]

Experimental Section

Chemicals

Tetraethylorthosilicate (TEOS, >99%), cetyltrimethylammonium bromide (CTAB, >99%), glycerol (>99.5%), methyltrimethoxysilane (MTES, >99%), and ammonium hydroxide solution (puriss. p.a., 25 wt% NH_3 in H_2O) were purchased from Sigma-Aldrich. All chemicals were of high purity and were used without any further purification.

Crude orange oil was kindly donated by Agrumi-Gel (Barcellona Pozzo di Gotto, Italy).

Material Synthesis

Glycerol (25 mL), CTAB (360 mg), and ammonium hydroxide (2.5 mL) were added to deionized water (250 mL) in a glass balloon kept under stirring at room temperature for 10 min after which the solution was added with crude orange oil (6 mL). The mixture kept in an ice-bath was sonicated by an ultrasonic processor (Bandeline, Sonoplus HD 4100) for 10 min at 25% amplitude. The resulting microemulsion kept at 40 °C under mechanic stirring (IKA Eurostar digital Werke) at 400 rpm was added dropwise with TEOS (16 mL), and left under stirring for 48 h. The white precipitate was filtered, washed extensively with deionized water and mildly dried in an oven (30 °C) overnight.

A blank sample was prepared with the same procedure substituting the orange oil with an equivalent volume of n-hexane. The synthesis of the methyl-modified silica microcapsules started from a similar solution of deionized water (250 mL), CTAB (3 g) and NH_4OH 25% (2.5 mL) kept under stirring at room temperature for 10 min and thus added with a solution of orange oil (6 mL) in cyclohexane (20 mL). The resulting emulsion was put in an ice-bath and sonicated by ultrasonic processor (Bandeline, Sonoplus HD 4100) for 10 min at 25% amplitude. The microemulsion was then transferred to a flask and kept under mechanical stirring at 400 rpm at 40 °C while a solution of TEOS (38 mL) and MTES (1.8 mL) was added dropwise. The white precipitate readily obtained was filtered, washed extensively with deionized water and dried in an oven (30 °C) overnight.

Characterization

The SiliaOrange essential oil loading was assessed by GC-FID analysis referring to the amount of limonene. A small amount (100 mg) of SiliOrange was added to a test tube along with 2 mL of 1 M NaOH aqueous solution in order to ensure complete dissolution of the silica shell. After stirring the mixture for 24 h, the non-polar fraction was extracted with 2 mL dichloromethane (DCM) and filtered through a syringe filter (Whatman PTFE, pore size 0,2 μm). The amount of limonene in the organic phase was measured via GC-FID analysis using undecane as internal standard. The analyses were carried out on a Shimadzu GC-FID 17A equipped with a Supelco SPB-1701 capillary column. TGA/DSC analysis was performed under nitrogen on a Mettler Toledo TGA/DSC1.

The orange oil maximum additive concentration (MAC) in CTAB was determined measuring the absorbance at 630 nm of the CTAB solution upon the addition of orange oil (0.025% to 1%) using a UV–VIS spectrophotometer (Shimadzu UV-1800) at 25 °C. The wavelength of 630 nm was chosen to avoid interference with the absorbance of orange oil.

Dynamic light scattering (DLS) and Zeta potential measurements were performed using a Malvern Zetasizer Nano ZS equipped with a He–Ne laser at a power $P = 4.0$ mW operating at 633 nm wavelength. The average diameter and size distribution (polydispersity index, PDI) of SiliOrange aqueous suspensions were determined by Photon Correlation Spectroscopy (PCS). Each solid sample was dispersed in filtered (0.2 μm) bi-distilled water. The instrument setting conditions were equal to those described above for the particle size measurements. Each sample was analyzed in triplicate.

The scanning electron microscope (SEM) pictures were obtained using a Zeiss EVO MA10 microscope equipped with an SE-Everhart–Thornley secondary electron detector. A lanthanum hexaboride cathode was used as the source of electrons. The accelerating voltage was 20 keV and probe current 10 pA. All imagines were acquired in ultra vacuum condition (10^{-7} mbar). To increase the conductivity of the sample, each material sample was deposited with an ultrathin layer

(2 nm) of gold nanoparticles using an AGAR Sputter Coater for gold.

Toxicological Bioassays

The biopesticide formulation used throughout this work was comprised of silica (SiO_2) SiliOrange microcapsules (0.5 g) dispersed in distilled water (150 mL).

Larvae of *S. littoralis* used for the bioassays were collected from the laboratory insect colony maintained in a climatic chamber (Refrigerated incubator model IRE-475, Raypa R. Espinar, S.L., Spain) at 24 ± 1 °C, 50 ± 10% R.H. and L12:D12 h photoperiod. *S. littoralis* larvae were reared on artificial diet according to a published method[14] inside plastic boxes (10 × 15 × 5 cm) that were covered with fine mesh to provide ventilation.

The rearing of *A. gossypii* was initiated from aphid infested organic greenhouse solanaceous crops inside ventilated propylene boxes (56 × 39 × 42 cm) offering zucchini plants (*Cucurbita* pepo L., cv "Bianca di Trieste") as hosts. Host plants were replaced twice a week and kept at the aforementioned controlled laboratory condition. For the insect rearing and the bioassays, zucchini plants were used at the phenological stage of 3rd true leaf grown in 2 L pots in greenhouse conditions. For the experiment, newly molted adults (24 ± 12 h old) were obtained by isolating 3rd instar nymphs on clean zucchini pots one week before the bioassay started. Nymphs were maintained at the same environmental laboratory conditions. The biological experiments were carried out at the Department of Agriculture, Food and Environment of the University of Catania (Italy).

Lethal and Sublethal Toxicity on Spodoptera littoralis

The contact toxicity and the consequent sublethal effect of the aqueous suspension of SiliOrange on *S. littoralis* larvae were evaluated by spraying same age larvae. For each replicate five to ten 2nd instar larvae of *S. littoralis* were topically sprayed with SiliOrange on an absorbent paper sheet and were then transferred to a plastic ventilated arena (4 × 5 × 2 cm) containing the artificial diet, provided *ad libitum*. Every 48 h the following biological parameters of insects were measured: i) larval survival (%), ii) development time (i.e., time elapsed, expressed in hours, from egg hatching to pupa formation), and iii) pupal weight using a 10 mg sensitive Mettler–Toledo microbalance.

An untreated control (distilled water), the blank of SiliOrange, and a treated control with the pyrethroid lambda-cyhalothrin (Karate Zeon, Syngenta Italia, a.i. 9.48%) at the highest label dose recommended for the control of noctuid moths in tomato protected crops (i.e., 0.125 L ha^{-1}), were used as controls. The boxes containing sprayed *S. littoralis* larvae were maintained in a climatic cabinet at the controlled climatic conditions reported above. Larval frass was removed every two days from the experimental arena, were cleaned up by and the factitious food was also renewed. Six experimental replicates were performed with the microencapsulated formulations and the controls.

<u>*Lethal and Sublethal Effect on Aphis gossypii*</u>

The contact toxicity of SiliOrange was evaluated on the survival and the progeny of *A. gossypii* by spraying young, same age adult aphids feeding on a zucchini plant. For this bioassay, twenty same age young adults feeding on a fresh 2-week old zucchini plant (that represented a single replicate) were sprayed from a distance of 0.3 m with a 0.1 L hand sprayer until runoff. A total amount of 1 g SiliOrange suspension was sprayed. Such methodology was chosen because it mimics a potential field scenario in which the chemical control of aphid pests is routinely pursued through insecticide foliar spraying devices (**Figure 1**).[15]

Sprayed aphid infested plants were left to dry under laboratory conditions and each plant was fixed with modelling-clay in a twosuperposed transparent plastic covered with a net[16] and kept inside a climatic cabinet at the previously described condition.

The aphid survival was assessed under a stereomicroscope 24 h after the beginning of the treatment. The aphid progeny (i.e., number of produced nymphs by females) was counted daily for ten consequent days. To compare the toxicity with a control and an established insecticide, an untreated control was used (i.e., distilled water), the blank of SiliOrange and the neonicotinoid Afidane 200 SL (Chimiberg, Italy, a.i. imidacloprid 17.7%) at the highest recommended concentration for controlling aphid pests in greenhouse crops (i.e., 0.75 L ha^{-1}). This bioassay was replicated five times per treatment group.

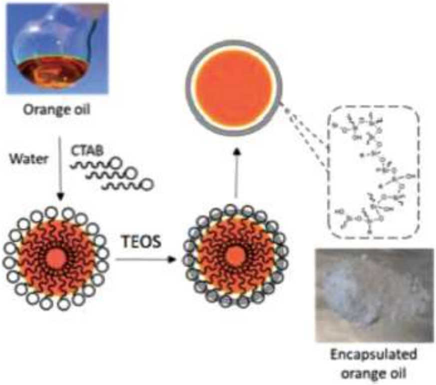
Figure 1. *Zucchini plant sample hosting Aphis gossypii insects prior to spraying the SiliOrange suspension that is in the spraying device on the right side*

Data Analyses

The homogeneity and normality of the dependent variables (i.e., measured biological parameters) were checked through Levene and Shapiro-Wilk tests and the dataset was log-transformed whenever needed. The effect of the independent variables (i.e., chemicals) on the biological parameters (i.e., larval survival, the developmental time and the pupal weight for *S. littoralis* and the adult survival and the progeny for *A. gossypii*) were tested by carrying out a single factorial ANOVA. Least Significant Difference (LSD) post hoc test was used for multiple means comparisons among the chemical treatments at $p \leq 0.05$. This statistical analysis was performed in RStudio (Version 4.0.0 – 2020).[17]

Results and Discussion

The template-driven sol–gel microencapsulation of orange oil displayed in Scheme 1 was successful, affording SiO_2 spherical microcapsules with a 7 wt% orange oil load, consistently assessed by means of GC. Though spherical and homogeneous, the methylated microcapsules had a modest 0.5 wt% content of essential oil (EO). This shows that the presence of the methylmodified silane during the sol–gel polycondensation favored the exit of the hydrophobic molecular components of the EO from the micellar structures, which is required for the entrapment of an hydrophobic substance in water (Scheme 1). We briefly remind that in the two-step sol–gel microencapsulation process of organic molecules in silica-based microcapsules from oil in water (O/W) emulsions, pioneered by Avnir and co-workers,[18] the emulsion droplets act as a microreactor environment for the hydrolysis and condensation reactions of Si alkoxides.



Scheme 1. *Synthesis of SiliOrange microcapsules: i) sonication of the essential oil in the presence of aqueous CTAB forms a stable microemulsion, ii) addition of TEOS forms the silica shell encapsulating the nanoemulsion core, iii) precipitation of the microcapsules followed by filtration and washing*

In the present case of SiliOrange synthesis, glycerol added to the precursor sol–gel mixture decreases the dielectric constant of the continuous phase thereby reducing self-aggregation of the cationic surfactant CTAB molecules via enhanced electrostatic interactions in an elegant way in which glycerol and CTAB molecules do not directly interact.[19] We thus measured the maximum additive concentration (MAC), namely the highest concentration of a lipophilic compound that can be incorporated into a micellar surfactant solution at a given surfactant concentration, of orange oil in CTAB to maximize the efficiency of sol–gel microencapsulation by assessing the amount of orange oil that was effectively solubilized by the surfactant (CTAB). **Figure 2** shows that CTAB micelle saturation occurs between 0.10% and 0.35% orange oil concentration, which leads to the rapid increase in the absorbance at 630 nm due to formation of un-dissolved orange oil droplets remaining dispersed in the aqueous phase. At this concentration micelles were no longer able to accumulate the oil in the core. This absorbance increase allowed us to determine 0.05 wt% as the ideal concentration of orange oil that ensures full microencapsulation of the EO volume (6 mL) added to the sol–gel precursor mixture (see the Experimental Section).

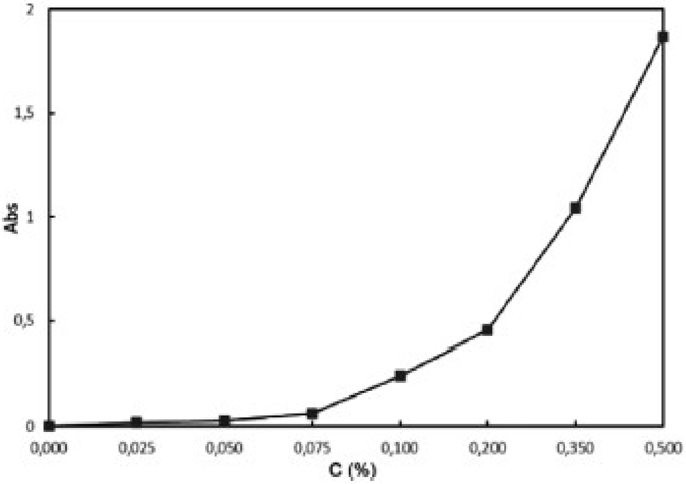


Figure 2. *Absorbance of CTAB micelles at increasing concentrations of orange oil*

The SEM photograph (**Figure 3**) clearly shows that SiliOrange is comprised of sub-micron spherical SiO_2 capsules of narrowly distributed size.

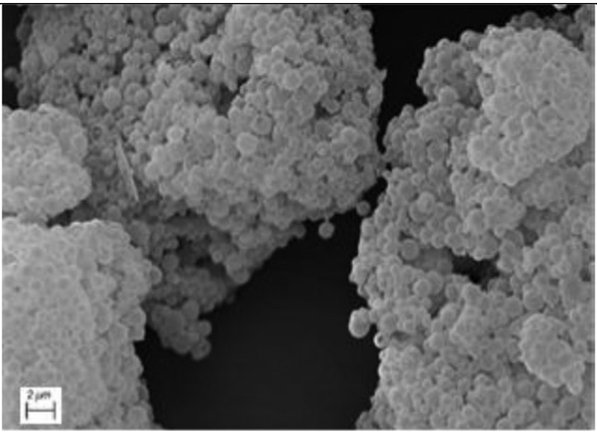


Figure 3. *SEM photograph of SiliOrange* SiO_2 *microcapsules having 7 wt% orange oil load*

The particle size distribution from dynamic light scattering (**Figure 4**, top), derived from a deconvolution of the measured intensity autocorrelation function of the sample using a nonnegatively constrained least squares fitting algorithm, pointed to a predominant population constituted of rather monodispersed microparticles of 0.153 μm diameter with a polydispersity index (PDI) of 0.249.

As an indirect measure of the net charge of ceramic particles in suspension, the zeta potential of silica microparticles measured in a dispersant indicates the stability of a suspension. Hence, the ζ (zeta) potential distribution (Figure 5.5, bottom) measured at the pH (6.3) determined by the simple addition of the microparticles to deionized water using the principles of laser Doppler velocitometry and phase analysis light scattering (M3-PALS technique) indicated good stability of aqueous suspensions of SiliOrange microspheres (34.5 mV).

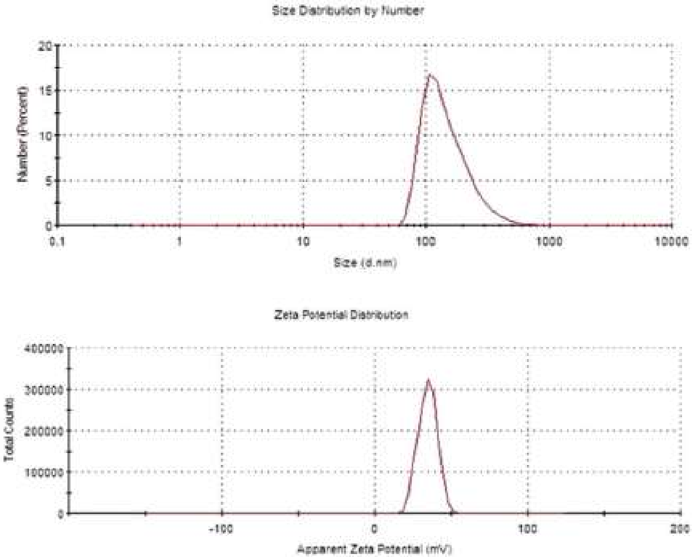


Figure 4. *Particle size distribution for SiliOrange SiO_2 microcapsules doped with 7 wt% orange oil (top); ζ potential distribution (bottom)*

The larger the absolute zeta potential value, the more stable the colloidal dispersion. A rule of thumb based on the Derjaguin, Landau, Verwey, and Overbeek theory suggests that values of ζ equal to or larger than ±30 mV are enough to promote stable water suspensions.[20] In light of practical applications, it is also relevant herein to notice that for amorphous silica, namely for glassy particles such as those comprising the SiliOrange material, the ζ potential rapidly decreases toward more negative values when increasing pH,[21,20] thus enabling the use of these microcapsules in aqueous systems of widely different pH.

The thermogravimetric analysis (TGA) describes the material's thermal stability and its fraction of volatile components. The plot shown in **Figure 5** indicates successful encapsulation of orange oil. In the blank sample, the first weight loss in the TGA was due to water evaporation (−6.18%) followed by a more significant weight loss peak at 300 °C likely due to water formed by condensation of silanol groups at the SiO_2 surface. Eventually, at 550 °C also the entrapped surfactant molecules were released and thermally decomposed.

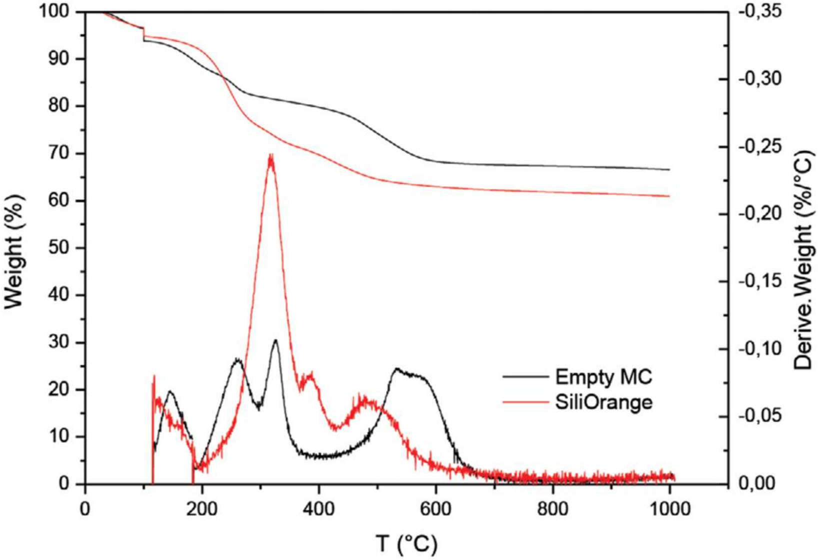


***Figure 5**. Top: TGA curves of empty (black curve) and SiliOrange microcapsules (red curve). Bottom: derivative thermogravimetry (DTG) plot*

The SiliOrange TGA profile is simpler. The significant weight loss (−32.15%) at around 300 °C following the same first weight loss due to water evaporation (−5.90%) was caused by concomitant orange oil and silanol groups condensation, similarly to what happened with core–shell silica microcapsules encapsulating glycerol (**Table 1**).[22]

T (°C)	SiliOrange weight loss (%)	Blank SiO_2 weight loss (%)
100	5.91%	6.19%
300	32.15%	13.25%
590	–	13.15%
	Total loss: 38.06%	Total loss: 32.59%

Table 1. *Weight loss in SiliOrange and empty* SiO_2 *microcapsules during the thermogravimetric analysis.*

A sample of SiliOrange (0.5 g) dispersed in water (150 mL) was tested as potential sustainable biopesticide against two insect herbivores having different feeding strategies. We chose the cotton leafworm *S. littoralis* as chewing pest and the cotton aphid *A. gossypii* as sap-feeding pest. In our standardized experimental setup, we chose larvae of similar age to exclude any potential effect of age on the observed biological parameters (i.e., mortality), as well as for ensuring the replicability of the results.

In the contact toxicity bioassay, the SiliOrange aqueous formulation used in this work reduced the survival of larvae by ≈40% in comparison to the untreated control ($F_{3,\ 22}$ = 45,17; p = 0.0001). Nevertheless, *S. littoralis* larvae sprayed with SiliOrange survived significantly longer than larvae treated with the pyretroid-based insecticide that caused ≈100% mortality (**Figure 6**).

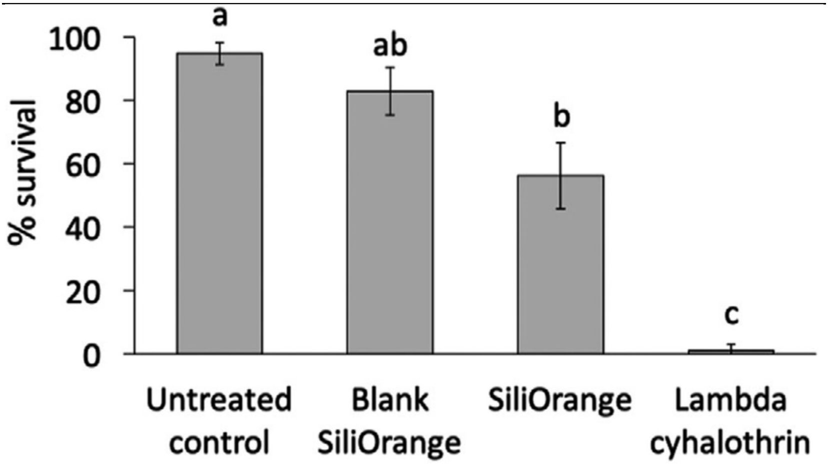


Figure 6. *Mean survival (±SE) of* Spodoptera littoralis *larvae in the contact toxicity bioassay. Bars with different letters indicate significant differences at $p < 0.05$ (ANOVA, LSD test)*

Conversely, no statistical differences were found between the survival of larvae sprayed with distilled water (i.e., untreated control) and the blank of SiliOrange. Similarly, treatment with SiliOrange significantly decreased the development time of *S. littoralis* larvae by 1.6 times in comparison to the untreated control ($F_{3,22} = 45,59$; $p = 0.0001$) (**Figure 7**).

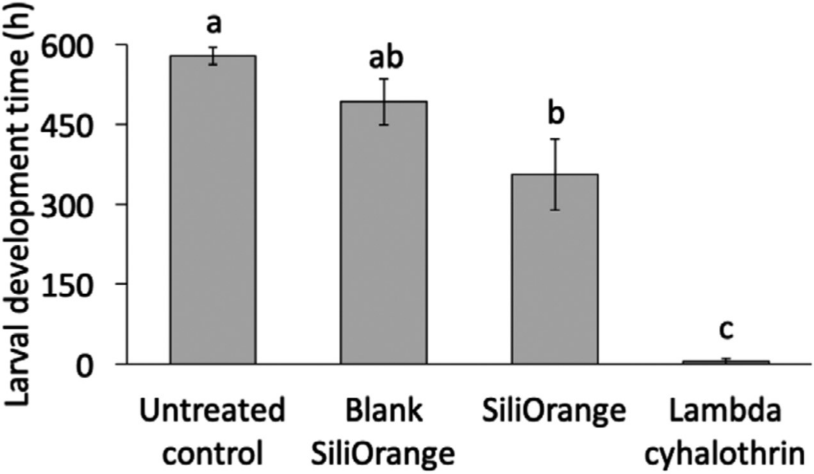


***Figure** 7. Mean development time (±SE) of* Spodoptera littoralis *larvae in the contact toxicity bioassay. Bars with different letters indicate significant differences at* $p < 0.05$ *(ANOVA, LSD test)*

As a consequence, also the pupal weight of *S. littoralis* was negatively affected by SiliOrange ($F_{2,\,17} = 6.86$; $p = 0.007$) (**Figure 8**, 192.11 ± 36.62 mg) compared to larvae sprayed with distilled water (578.07 ± 16.16 mg). Pupal weight was not determined in the treated control (i.e., lambda-cyhalothrin), because none of the sprayed larvae survived this bioassay.

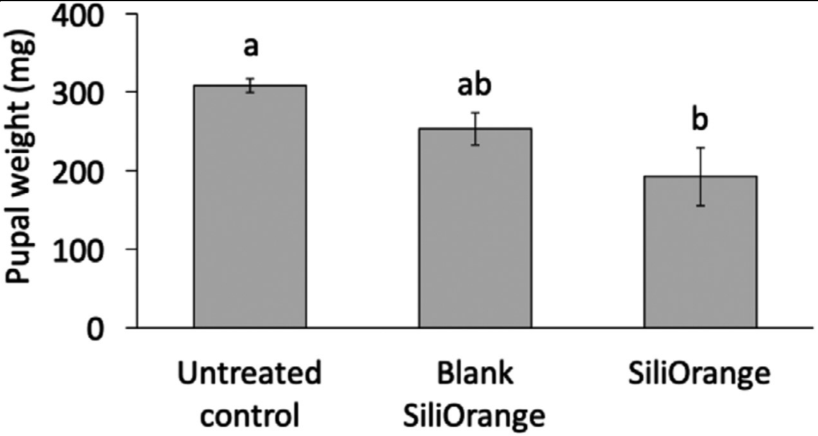


Figure 8. *Mean weight (mg) (±SE) of* Spodoptera littoralis *pupae formed from larvae sprayed in the contact toxicity bioassay. Bars with different letters indicate significant differences at p < 0.05 (ANOVA, LSD test)*

The entomotoxicity of silica-based nanoparticles per se has been proven against different lepidopteran pests[23,24,25] including *S. littoralis*.[26,27] Similarly, the insecticidal activity of citrus essential oils has been demonstrated in several lepidopterans including the cotton leafworm.[5,28,29]

Although a limited number of studies proved the toxicity of silica nanostructures prepared by the sol–gel method on *Spodoptera* spp.,[23,30] no information is currently available on the toxicity of silica nanoparticles encapsulating *Citrus sinensis* essential oil against *S. littoralis*. Silica nanoparticles can be physiosorbed by the insect cuticular lipids causing mostly mechanical damages that lead to death.[31] Furthermore, the terpenes contained in Citrus essential oil can cause insect mortality through multiple modes of action.[32] SiliOrange at the tested ultralow amount caused no acute toxicity in *A. gossypii* adults, whereas the imidacloprid-based treated control caused the highest mortality ($F_{3,20}$ = 233.84; $p = 0.001$) (**Figure 9**).

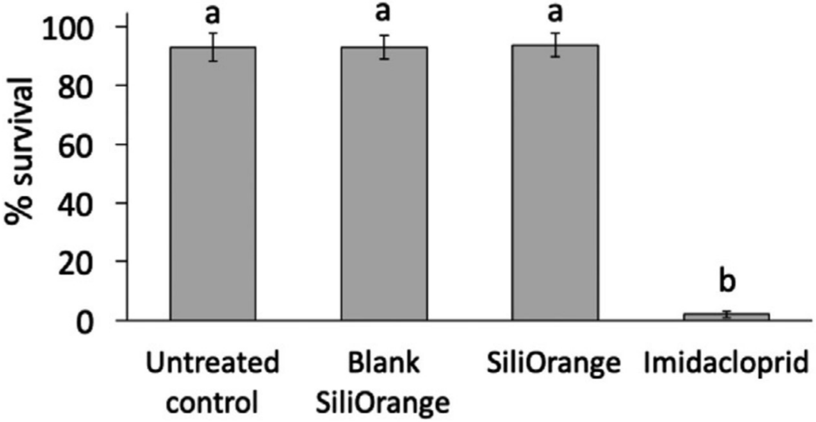


***Figure 9**. Mean survival (±SE) of* Aphis gossypii *adults in the contact toxicity bioassay. Bars with different letters indicate significant differences at $p < 0.05$ (ANOVA, LSD test)*

This absent toxicity of SiliOrange microcapsules in *A. gossypii* bioassay using the ultralow amount of encapsulated crude orange oil might be explained by the different susceptibility of the pest to the citrus essential oil encapsulated in the nanoparticles.[33] Moreover, considering that *Citrus sinensis* is a host plant of *A. gossypii*, a possible insect adaptation to the secondary metabolites of the host plant may have reduced the susceptibility of this species to citrus essential oil constituents as a result of a co-evolutionary process.[34]

By contrast, SiliOrange significantly decreased the offspring by ≈48% in *A. gossypii* in comparison with the untreated control ($F_{3,20} = 4.08$; $p = 0.025$). However, the same depletion in the progeny production was found in the SiliOrange group and its blank control (**Figure 10**). This reduction of aphid fertility in both silica-based treatments may be caused by the action of amorphous silica gels through contact leading to desiccation as in different agricultural pests, such as the black bean aphid *A. fabae* Scopoli (Hemiptera: Aphidiidae).[35]

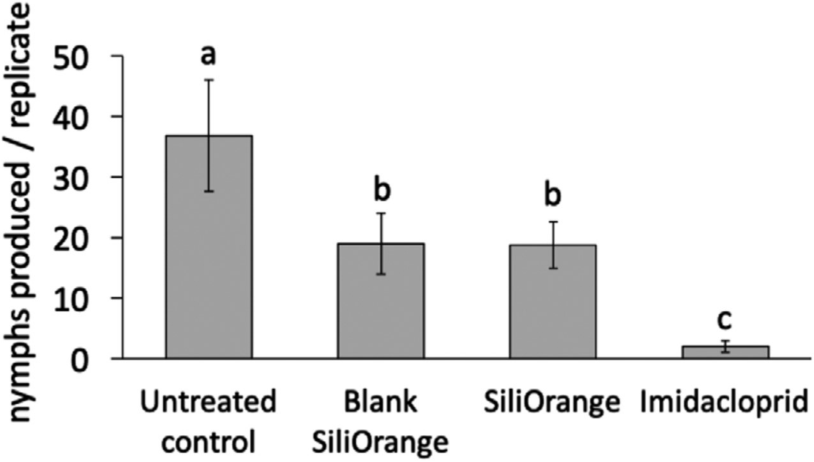


Figure 10. *Mean number (±SE) of* Aphis gossypii *progeny produced by sprayed female adults in the contact toxicity bioassay. Bars with different letters indicate significant differences at* $p < 0.05$ *(ANOVA, LSD test)*

Outlook and Conclusions

This work reports a reproducible synthetic sol–gel route to relatively monodispersed (PDI = 0.249) spherical SiO_2 submicron particles (153 nm in size) loaded with 7 wt% crude orange oil (SiliOrange), and its first application as a potential formulation for the control of economically relevant agricultural pests with different feeding strategies.

We have demonstrated for the first time that an ultralow amount of SiliOrange capsules dispersed in water show surprisingly high insecticidal activity against the cotton leafworm *S. littoralis*, and significantly reduce the number of offspring of cotton aphid *A. gossypii* under laboratory testing conditions. The tested water-based formulation contained 500 mg of SiliOrange microspheres in 150 mL water. This translates into 35 mg of crude orange oil only, which equates to a nominal concentration of 0.23 ppm of orange oil in water. The activity of this highly diluted dispersion was compared to that of commercially available insecticides with well-known active ingredients.

From the viewpoint of pesticide safety, the entomotoxicity of SiliOrange toward *S. littoralis* as well as its environmental impact at different levels should be assessed in the field.[36] Our research also revealed that SiliOrange sub-micron silica capsules obtained through the sol–gel process[37] can impair the fertility of *A. gossypii*. Therefore, further studies on the sublethal impact of the chemical at transgenerational level should be investigated by the estimation of population demographic indexes.[38–40]

Although preliminary studies on the toxicity of nanomaterials against insect pests have been reported,[41] current knowledge on their mechanisms of action and physicochemical characteristics in open field conditions is still limited, thus new investigations are urgently needed.[40]

Regardless of the commercial importance of orange essential oil,[1] including its use as active ingredient in new biobased insecticides,[9] successful sol–gel microencapsulation in spherical SiO_2 particles has not been reported so far. Previous work synthesized silica microparticles functionalized with limonene,[42] or silica and organosilica microcapsules doped with bergamot oil.[43] Though similar, the aforementioned preparative route cannot be applied to the microencapsulation of orange oil, which required the crucial presence of glycerol in the sol–gel precursor mixture, likely to reduce the aggregation of the surfactant CTAB molecules in the micellar nanoreactors (Scheme 5.2).[19,37]

For the purpose of pest control using SiliOrange microcapsules, two most important aspects are the safety for human health[44] and the environmentally friendly nature[8] of amorphous mesoporous SiO_2. Both aspects are promising for the approval of the SiliOrange material as solid pest control agent.

Considering the ease of the preparation of the material, the biocompatibility of both silica and orange essential oil, and the ultralow amount of SiliOrange microcapsules used, these results may open the route to sustainable pest control based on sol–gel microencapsulated orange oil.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

All raw data concerning the biological parameters represented in the graphs of this article are available upon contacting one of the corresponding authors (L.Z.). All data concerning the characterization of the SiliOrange materials are available upon contacting one of the corresponding authors (R.C.).

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5.2 Nanostructured lipid carriers of essential oils as potential tools for the sustainable control of insect pests

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Nanostructured lipid carriers of essential oils as potential tools for the sustainable control of insect pests

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Highlights

- Nanostructured Lipid Carriers (NLCs) of three Essential Oils (EOs) were developed
- EO-NLCs were characterized for their stability and particle size
- Their efficacy was tested towards three insect pests with various feeding strategies
- NLCs can successfully deliver EOs by keeping unaltered their properties
- EO-NLCs are able to provide plant insect protection as alternative sustainable tools

Introduction

Plant essential oils (EOs) are highly volatile substances with complex chemical structure mostly constituted by aromatic compounds that smell intensely (Regnault-Roger et al., 2012). They are synthesized in different plant organs and structures (e.g., flowers, leaves, trichomes) and, stored in secretory cells and epidermal glands (Bakkali et al., 2008). EOs play many different roles in plant-arthropod interactions (e.g., attract pollinators, mediate trophic activity of insect pests and natural enemies) and show antimicrobial and insecticide activities (Soares et al. 2019; Al-Ansari et., 2021; Benelli et al., 2021; Du et al., 2021; Karkanis & Athanassiou 2021; López et al., 2021). As a consequence, EOs have a key role in plant protection and can be used as alternative tools to reduce the negative environmental impact of conventional management systems (Giunti et al., 2019; Pavela et al., 2020; Andrade et al., 2021; Sciortino et al. 2021).

Unfortunately, EOs have intrinsic physicochemical characteristics that are difficult to manage and do not allow their practical use (Tian et al., 2021). For instance, EOs can cause toxicity on plants when formulated at high concentrations, but their efficacy is reduced in the field due to high degradation patterns. Moreover, EO homogeneous solutions in water are difficult to obtain and their stability is often complicated (Campolo et al., 2017). Nevertheless, these drawbacks could be easily overcome by nanotechnologies that are currently used in agriculture as bio-active formulations (Athanassiou et al., 2019; Pascoli et al., 2020; Falleh et al., 2021; Pavela et al., 2021).

Nanostructured Lipid Carriers (NLCs) are a second-generation nano-carriers constituted by a blend of a solid lipid with a liquid lipid that cover the deficiencies of solid lipid nanoparticles (SLNs) which are solely made by solid lipids (Müller et al., 2000). This new technology ameliorates the physical and colloidal stability of formulation and its resistance to ultraviolet light (UV) and oxidation (Nguyen et al., 2012). NLCs could be thus a promising tool to encapsulate EOs because this delivery system can control their release after the application, protect them from degradation and lower their toxicity (Katopodi & Anastasia, 2021). Many studies have shown interesting results for EOs encapsulation in NLCs and their properties are well known and routinely used in pharmaceutics, food industry and cosmesis (Maroofpour et al., 2019; Dobreva et al., 2020; Waghule et al., 2020). In agricultural crop protection, NLCs could help in reducing insecticide doses and avoid their repeated applications. However, only few studies have reported the use of NLCs as pesticide formulations and new advances in this field are thus required (Frederiksen et al., 2003; Nguyen et al., 2012; 2016).

In this framework, the aim of the study was the development of potential bio-insecticide products made by long-term stable NLCs loaded with commercially available EOs of Rosemary, Lavender and Peppermint as intrinsic oily components (Carbone et al., 2018; Bonaccorso et al., 2021). We tested under laboratory conditions the efficacy of EO-NLCs in term of lethal and sublethal effects against three agricultural key pests with three different feeding strategies. The sap-sucking, *Aphis gossypii* Glover (Hemiptera: Aphididae), the chewer, *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) and the leafminer, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) were chosen because of their economic relevance in agricultural context (Campolo et al. 2014; Biondi et al., 2018; Hullé et al., 2020; Miranda-Fuentes et al., 2021; Desneux et al., 2022). The results of this study could contribute providing the potential basis for the sustainable management of agricultural pests in cropping systems.

Materials and methods

EO-NLCs

Materials

Hydrogenated Coco-Glycerides (Softisan 100) was purchased from IOI Oleo GmbH (Oleochemicals, IOI group). Oleoyl Macrogol-6 Glycerides (Labrafil) was a gift from Gattefossé Italia s.r.l. (Milano, Italy). Kolliphor RH40 was bought from BASF Italia S.p.a. (Cesano Modena, Italy). Rosemary (*Rosmarinus officinalis*) (monoterpenes 27.68 ± 0.001 %; oxygenated monoterpenes 68.34 ± 0.015 %), Lavender (*Lavandula angustifolia*) (monoterpenes 2.64 ± 0.000 %; oxygenated monoterpenes 89.87 ± 0.020 %) and Peppermint (*Mentha x piperita*) (monoterpenes 9.27 ± 0.000 %; oxygenated monoterpenes 80.43 ± 0.051 %) EOs were purchased from Esperis s.p.a. (Milan, Italy). The tested EOs have been chemically characterized by Campolo et al. (2020a). Triglyceride caprylic-capric (Tegosoft CT, Miglyol 812) was supplied by Farmalabor (Canosa di Puglia, Italy).

Nanoparticles Preparation

NLCs were prepared through phase inversion temperature (PIT) method (Carbone et al., 2014). Both water and lipid phase – composed of surfactants Kolliphor RH40 (6.0% w/v), Labrafil (7.5% w/v) and the solid lipid Softisan (10.0% w/v) – were separately heated to 70°C. Then the lipid phase was added with the EO (10.0% w/v). In order to obtain the nanoparticles, the aqueous phase was added dropwise. The mixture was kept under stirring for 1 h at 25°C, obtaining nanoformulations containing Lavender (L-NLC), Peppermint (P-NLC) and Rosemary (R-NLC) EOs. Moreover, a control formulation without EO was prepared using Tegosoft (CT-NLC). All the samples were purified removing the excess of surfactants through ultracentrifugation (SL16R Centrifuge, Thermo Scientific, Rodano, Italy) at 1°C and 13000 rpm for 2 h. The obtained pellets were vortexed (Heidolph Reax 2000, VWR, Milan, Italy) for 60s in order to resuspend the nanoparticles.

Photon Correlation Spectroscopy (PCS)

Photon Correlation Spectroscopy (PCS) is a hydrodynamic technique that allows the quantification of translational and/or rotational diffusion coefficients, related to the size and shape of nanoparticles. The light ray passing through the particle dispersion is diffused with an angle that depends on the relative positions of the particles in relation to the direction of arrival of the ray. Due to Brownian motion, particles position continuously fluctuate thus causing a variable scattering signal, which reaches the detector (a photomultiplier or an avalanche photodiode). The diffused light scattering is measured as a function of time and the digitalized to an autocorrelator (Pecora, 2000) In order to perform this analysis, a Zetasizer Nano S90 (Malvern Instruments, Malvern, UK) was used, with a detection angle of 90°, a temperature of 25 °C and a 4 mW He–Ne laser operating at 633 nm.
The same instrument was also used to analyse the zeta potential (ZP) of NLCs, through the measurement of electrophoretic mobility. This parameter is calculated by the quantification of the velocity of sample particles using Laser Doppler Velocimetry technique. Since the presence of superficial charge promotes electrostatic repulsions between sample particles, thus preventing their aggregation, the determination of ZP is a key analysis to estimate stability of colloidal suspensions. Twenty-four h after the preparation, mean particle size (Zave), polydispersity index (PDI) and ZP of the diluted samples (1:10 in deionized water) were measured. The obtained values were reported as the mean of at least three measurements ± standard deviation (SD).

Stability Studies by Turbiscan® AG Station

Turbiscan® AGS (Formulation, L'Union, France) is an apparatus composed of an Aging Station, with a robot and three thermoregulated blocks for the storage of the samples, and a Turbiscan Lab Expert, which uses a multiple light scattering to analyse light dispersion through a concentrated suspension. The infrared light source has a wavelength of 850 nm; the synchronous transmission (Tr) detector receives the light ray that crosses the sample at 180°, while the back scattering (BS) detector receive the refracted light with a 45° angle from the incident ray. Every scan produces 1625 results since the transmission data are collected every 40 µm through the full cell height (65 mm). This instrument allows detecting any instability phenomena occurring in the sample during the storage. In particular, change in the volume fraction of the particles highlights migration phenomenon, while variation in particle diameter reveals coalescence phenomenon; as an output, variation of T and BS signals are obtained. The comparisons between the formulations are based also on Turbiscan Stability Index (TSI), which provides the numerical quantification of the general behaviour of the formulation. Because in earlier studies the importance of this apparatus in the assessment physical stability of nanoformulations (Carbone et al., 2020; Bonaccorso et al., 2021), a 30-days analysis was carried on at 25.0 ± 1.0 °C on 20 mL of each prepared NLC.

Biological materials

Three insect species having different feeding strategies, the sap-sucking *A. gossypii,* the chewer *S. littoralis* and the leafminer *T. absoluta*, were used for the experiments. Insect colonies were established in 2008 from individuals collected in protected vegetable crops in Ragusa (Italy) and breed twice a year with new specimens to reduce the genetic drift. Laboratory insect rearings were located in separated chambers at the Department of Agriculture, Food and Environment of the University of Catania (Italy) and kept at controlled environmental conditions, as follows: 24 ± 2 °C, 50 ± 10 % R. H., photoperiod of L14:D10 for *A. gossypii* and *T. absoluta* and L10:D12 for *S. littoralis*. *Aphis gossypii* was maintained on zucchini plants, *Cucurbita pepo* cv "Bianca di Trieste" (Cucurbitaceae), according to Ricupero et al. (2020). *Spodoptera littoralis* was reared on artificial diet in ventilated and screened plastic boxes (10 x 15 x 5 cm) following the methodology proposed by Di Lelio et al. (2019), *Tuta absoluta* was reared on tomato, *Solanum lycopersicum* cv "Creativo" (Solanaceae), as described by Zappalà et al. (2012).

For the experiment, newly moulted (12± 6 h-old) *A. gossypii* adults were obtained by isolating, seven days before the experiment coetaneous 3rd instar nymphs from the rearing on clean zucchini pots. *Aphis gossypii* isolated nymphs were kept at the same standardized laboratory conditions. Second instar larvae of *S. littoralis* and *T. absoluta* were isolated from the rearing.

Zucchini plants at the phenological stage of 3rd true leaf grown in 2 L pot were used in the bioassay for *A. gossypii*. Tomato and sweet pepper, *Capsicum annuum* cv "Grossum" (Solanaceae), plants that reached the phenological growth stage of 5th true leaf on main stem unfolded (BBCH-id 105) (Feller et al., 1995), were used in the bioassays for *T. absoluta* and *S. littoralis*, respectively. Plants were grown in squared pots (10 × 10 ×15 cm) with a mixture of topsoil (Gramoflor®, GmbH & Co. KG) and vermiculite (VIC, Italiana®) in greenhouses (200 × 300 × 300 cm) at natural environmental conditions and fertilized with Greenleaf 20.20.20® (Biolchim s.p.a., Italy) every fifteen days.

Toxicological bioassays

The NLC formulations used throughout the following toxicological bioassays consisted of NLCs dispersed in distilled water in the ratio 30:70 v/v. Experimental arenas were kept in climatic chambers at the same controlled environment conditions described for the insect laboratory rearing. All the bioassays were carried out at the Di3A of the University of Catania.

Aphis gossypii

The contact toxicity of EO-NLCs was evaluated on the survival and the progeny of *A. gossypii* by spraying coetaneous and young (0-2 d old) adults. For this bioassay, each single replicate was represented by twenty adults feeding on a pesticide-free zucchini plant that were sprayed following the methodology described by Sciortino et al., (2021). The mortality of *A. gossypii* sprayed individuals was assessed under the binocular after 24 hours. The total aphid progeny per replicate (i.e., number of produced nymphs by females) was counted daily for ten consequent days after the beginning of the EO-NLC exporsure. The toxicity of EO-NLCs was compared with an untreated control (i.e., distilled water), the CT-NLC and a treated control (Afidane® 200 SL, Chimiberg, Italy, a.i. imidacloprid, at the label rate of 750 ml/ha recommended against aphids on cucurbit crops). Per each tested EO-NLCs and the controls the bioassay was replicated five times.

Spodoptera littoralis

The toxicity of EO-NLCs was evaluated in terms of lethal and sublethal effect on *S. littoralis* by topical and ingestion routes of exposure. The lethal effect was assessed on the larval survival while the sublethal effect was evaluated on the feeding activity of larvae by measuring the eroded leaf area (mm^2). For ingestion treatment, the leaves were cut from healthy and pesticide-free sweet pepper plants, and leaf discs were made. Each EO-NLC solution was sprayed on the leaf discs and let dry in laboratory conditions. Sprayed sweet pepper leaf-discs were placed in a plastic box and five 2nd instar *S. littoralis* larvae were posed directly on the leaf discs allowing them to feed.

For the topical exposure, the leaves were cut from healthy and pesticide-free sweet pepper plants, and leaf discs were made and placed in the plastic box. Five 2nd instar larvae of *S. littoralis* were sprayed with the same solutions in an absorbent paper sheet as described above and then released on leaf-discs allowing them to feed. An untreated control (i.e, distilled water), a treated control (Karate Zeon®, Syngenta Italia S.p.A., a.i. lambda-cyhalothrin, at the label rate of 125 ml/ha recommended against lepidopteran pests on solanaceous crops) and the CT-NLC were included to compare toxicity of EO-NLC solutions. Per each exposure route, each tested EO-NLCs and the controls the bioassay was replicated five times.

Tuta absoluta

The toxicity of EO-NLCs was evaluated in terms of lethal and sublethal effect on *T. absoluta* by topical and ingestion routes of exposure. The lethal effect was assessed on the larval survival while the sublethal effect was evaluated on the feeding activity of larvae by measuring the eroded area (mm^2) of tomato leaves.

For the ingestion treatment, two 2nd instar larvae of *T. absoluta* were released to feed on treated plant substrate placed in a two-cup experimental arena (Biondi et al., 2012). Tomato pesticide-free leaves cut from healthy plants were sprayed with the EO-NLC solutions and let dry in laboratory conditions. Treated leaves were thus fixed in the arena and two coetaneous 2nd instar *T. absoluta* larvae from the rearing were placed on the leaves with a soft paintbrush.

For topical treatment, two 2nd instar larvae of *T. absoluta* were sprayed with the EO-NLC solutions through a hand sprayer on absorbent paper sheet (to prevent larval drowning caused by excess of solution), thus were moved with a paintbrush onto fresh and untreated tomato leaves cut from healthy plants previously placed in the two-cup experimental arena.

For both the exposure routes, the larval survival was recorded every 24 h for three days and the feeding activity was assessed by measuring the eroded leaf area through the image acquisition process at the end of each bioassay. Leaves were scanned with a high-resolution Epson® Perfection 4180 Photo in JPG format with 800 dpi of resolution. The resulting images were imported into Adobe Photoshop® (Adobe System Inc. 1990-2018) and a scale by a digital ruler was set as 318pixel = 10mm. With "Magnetic lasso" implement the eroded leaf area was manually selected and the data were collected. The same scale was used for all the replicates.

An untreated control (i.e., distilled water), a control treated with a commercial insecticide of known efficacy in controlling *T. absoluta* (Steward®, FMC Agro Italia Srl., a.i. indoxacarb, at the label rate of 125 g/ha recommended against lepidopteran pests on solanaceous crops) and the CT-NLC were included to compare the toxicity of EO-NLC solutions. Per each exposure route, each tested EO-NLCs and the controls the bioassay was replicated five times.

Data analyses

The data on lethal and sublethal effects of each EO-NLC formulation and control treatments were evaluated on the survival of the three target species, on the progeny of *A. gossypii* and on the feeding activity of *S. littoralis* and *T. absoluta*. Dataset of dependent variables was checked for homogeneity and normality of variance through Levene and Shapiro-Wilk tests and log-transformed if needed.

The progeny, the survival and the feeding activity of larvae (mm^2) for each dataset independently was subjected to one-way ANOVA analyses and the post hoc analyses were carried out using LSD test ($p \leq 0.05$). Statistical analysis was conducted on RStudio® (Version 1.1.463 – 2009-2018, Inc.).

EO-NLC characterization

After 24 h from the preparation through PIT method, the produced NLCs were characterized in terms of mean particle size and polydispersity index using PCS. The obtained results, reported in Table 5.13, show the presence of the EOs in NLC formulation allowed obtaining smaller nanoparticles compared to control CT-NLC. In particular, all EO-NLCs showed the presence of homogeneous nanoparticles of about 200 nm, with a single peak in size distribution as confirmed by PDI values lower than 0.3 (Carbone et al., 2018). This consideration suggests that Lavender, Peppermint and Rosemary EOs are able to provide a better organization of raw materials at the nanoparticles interface, compared to the commercial oil, therefore inducing the formation of nanosuspensions with greater homogeneity. ZP results highlight the presence of a negative superficial charge in all the samples, with similar values for Lavender and Peppermint NLCs, and for Rosemary and CT-NCLs. Because repulsion between particles with the same superficial charge prevents aggregation phenomena, the analysis of ZP values could suggest that R-NLC and CT-NLC are more promising in terms of long-term stability. On the other hand, also homogeneity has to be taken into account since it plays a key role in determining stability: CT-NLC showed PDI values > 0.3, thus it is possible to predict possible instability phenomena occurring in this formulation (Danaei et al., 2018).

Sample	Zave ± SD (nm)	PDI ± SD (a.u.)	ZP ± SD (mV)
L-NLC	198.9 ± 12.3*	0.216 ± 0.009*	-10.8 ± 1.3
P-NLC	179.85 ± 7.4*	0.207 ± 0.028*	-10.8 ± 0.9
R-NLC	156.0 ± 1.4*	0.251 ± 0.026*	-15.2 ± 0.8
CT-NLC	284.5 ± 59.3	0.480 ± 0.085	-15.4 ± 2.1

Table 5.13. *Mean particle size (Zave, nm) and Polydispersity Index (PDI) ± standard deviation (SD) of the prepared Lavender NLC (L-NLC), Peppermint NLC (P-NLC) and Rosemary NLC (R-NLC). * Significance for $p < 0.05$, comparison between EO-loaded NLC (L-NLC, P-NLC, and R-NLC) and the control NLC (CT-NLC)*

All NLCs were stored in Turbiscan® at 25°C for 30 days. The destabilization kinetics showed a significant different stability between control formulation CT-NLC and EO-loaded NLCs: after 4 weeks of storage, TSI value for CT-NLC are about 26-fold increased, while for L-NLC and P-NLC are about 15-fold increased (Fig. 5.14). Based on TSI profiles reported in Figure 1, the most stable formulation results to be R-NLC. In particular, the following stability scale could be defined: R-NLC > L-NLC > P-NLC > CT-NLC.

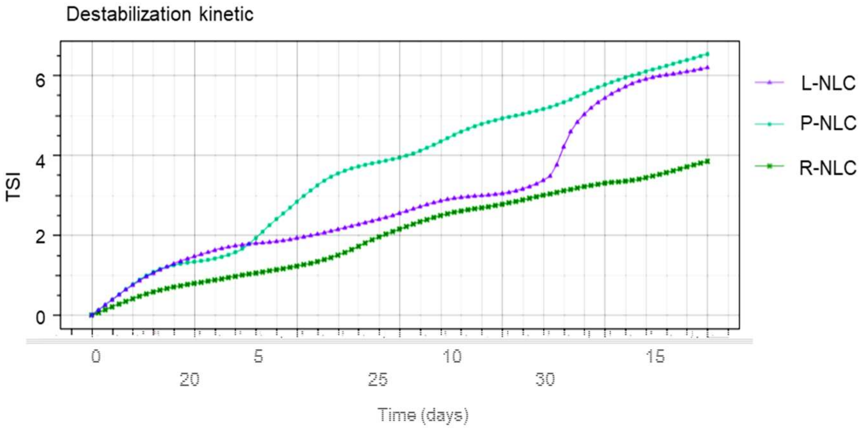


Figure 5.14. *Destabilization kinetics represented in terms of evolution of Turbiscan® Stability Index (TSI) of NLCs prepared with Lavender, Peppermint or Rosemary stored 30 days at 25 ± 0.5 °C*

From backscattering profiles, it is also possible to establish the type of instability occurring in the formulations (Fig. 5.15). In particular, all the samples showed a migration of the nanoparticles to the bottom of the cuvette, which is slight for EO-NLCs and emphatic for CT-NLC. However, for EO-NLCs this phenomenon is not significant since the variation in backscattering (ΔBS) was lower than 15%, while for CT-NLC it emerged also a sedimentation phenomenon associated to particles aggregation (ΔBS > 20%) as predicted by the high PDI value. As previously reported (Carbone et al., 2018), the phase separation which occurs during the storage is reversible, since it is possible to resuspend the nanoparticles through agitation.

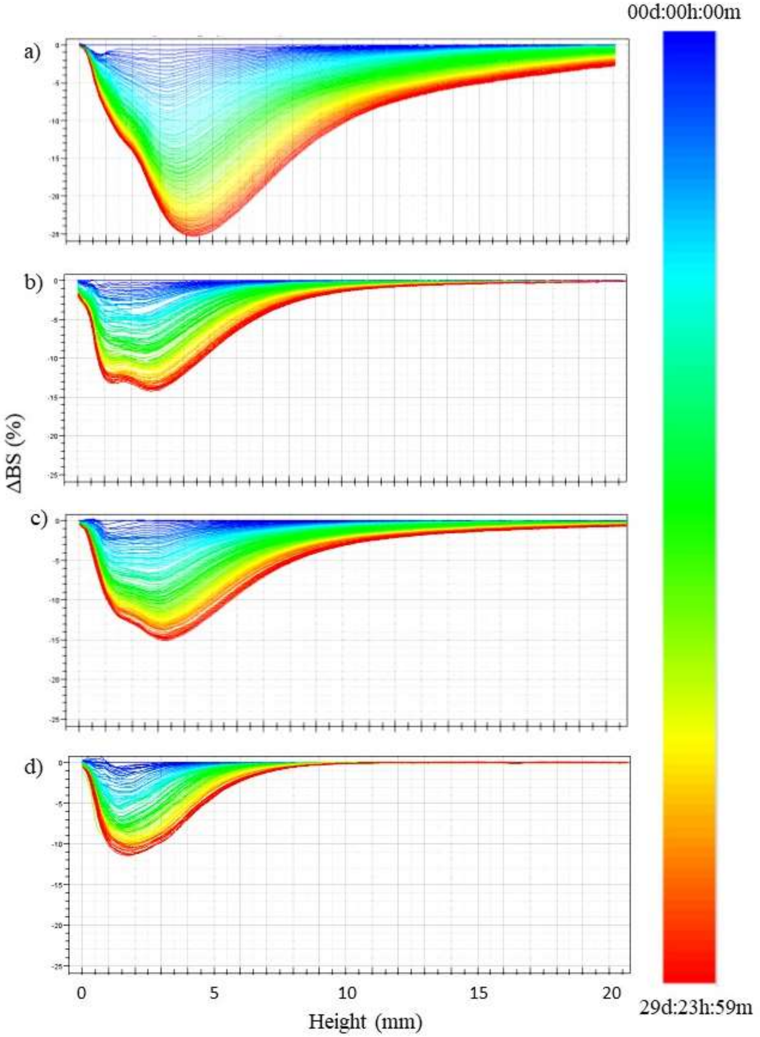


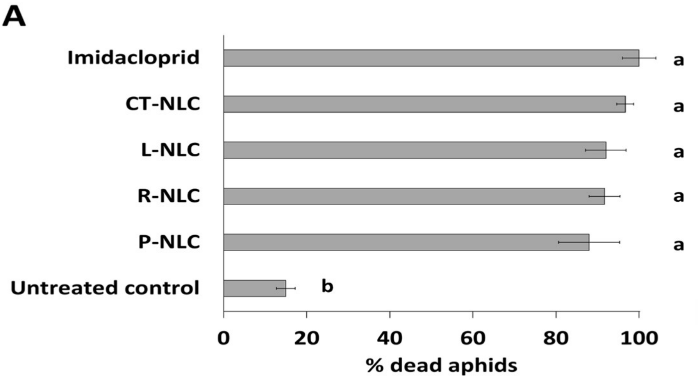
Figure 5.15. *Backscattering profiles (ΔBS) of CT-NLC (a), L-NLC (b), P-NLC (c) and R-NLC (d) stored in Turbiscan® for 30 days at a temperature of 25.0 ± 1.0 °C. Data are presented as a function of time (0–30 days) of sample height (0 to 20 mm) (the direction of analysis time is indicated by the arrow)*

Detailed results obtained from destabilization kinetics analysis (TSI values), reported in Figure 1, were in line with the affirmations above. In particular, in the first few days some similar instability phenomena occurred in L-NLC and P-NLC, contrarily from R-NLC. After 1 week of storage until 3 weeks, L-NLC and R-NLC behaviours were quite similar, while a significant particle migration to the bottom of the cuvette was detected for P-NLC. During the last week, L-NLC showed a great increase in TSI value reaching P-NLC curve, while R-NLC profile remained quite stable. Considering the first 3 weeks storage, the good stability of R-NLC and L-NLC could be related to the presence of smaller particles and great homogeneity, compared to P-NLC.

Toxicological bioassays

For *A. gossypii*, the sap-sucking pest tested in this study, the mortality and progeny of adults sprayed topically with EO-NLCs was assessed. For *S. littoralis* and *T. absoluta* the chewer and the leafminer pests respectively, larval survival and mean eroded leaf area (mm^2) of larvae topically sprayed and larvae developed on sprayed leaves with EO-NLCs were assessed.

Aphis gossypii



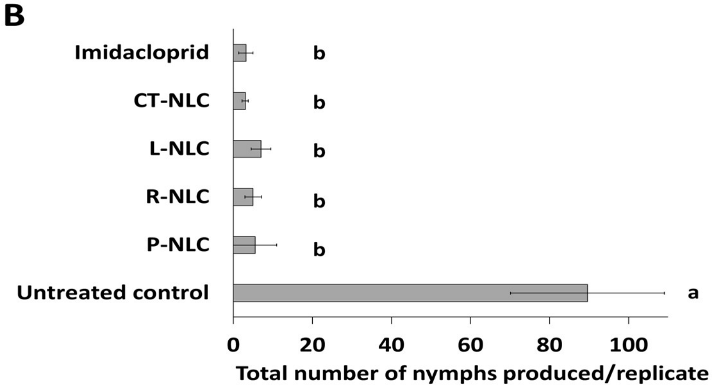


Figure 5.16. *Mean mortality (±SE) (A) and mean progeny (±SE) (B) of* Aphis gossypii *adults sprayed topically with EO-NLCs, control NLC and treated control with a commercial synthetic insecticide. Bars with different letters indicate significant differences at* $p < 0.05$ *(ANOVA, LSD post hoc test)*

NLCs with Rosemary, Lavender and Peppermint EOs caused a significant high mortality by topical exposure in *A. gossypii* after 3 days, in comparison to the untreated control ($F_{5,30} = 62.16$, $p<0.0001$) (Fig.5.16A). Also, the progeny of *A. gossypii* females after topical exposure with EO-NLCs decreased significantly in comparison to the untreated control ($F_{5,30} = 12.94$, $p<0.0001$) (Fig.5.16B).

Interestingly, CT-NLC alone caused significant mortality and decreased the offspring in *A. gossypii* similarly to the treated control (Afidane® 200 SL a.i. imidacloprid). This means that the high mortality and the reduction of aphid fertility can be due to the developed CT-NLC formulation that includes surfactants as main components. The toxicity of surfactants against aphid pests is supported by different studies (Wolfenbarger et al., 1967; Imai et al., 1994; Wood et al.,1997) because they penetrate insect cuticle by disrupting cell membranes, causing thereby the desiccation of soft-bodied insects (Kraiss & Cullen, 2014).

Spodoptera littoralis

Ingestion exposure

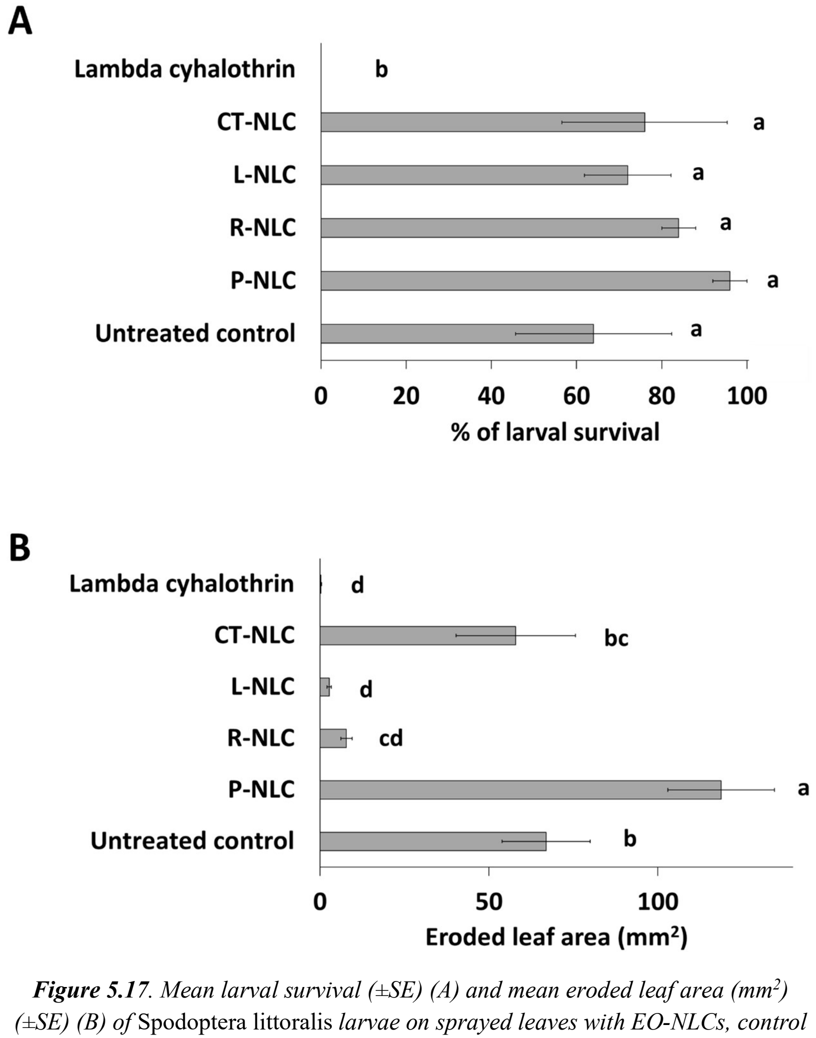
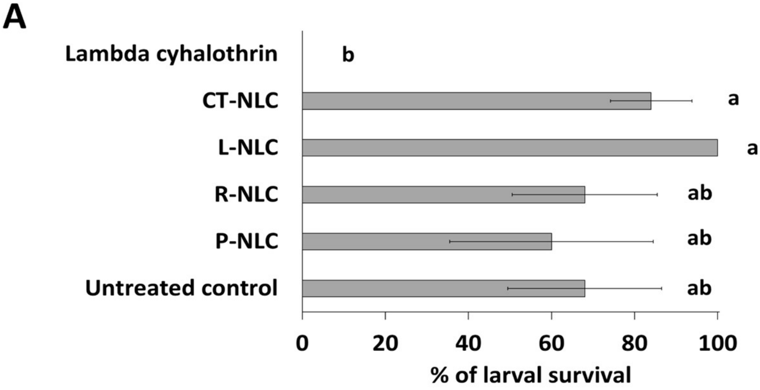


***Figure 5.17**. Mean larval survival (±SE) (A) and mean eroded leaf area (mm^2) (±SE) (B) of* Spodoptera littoralis *larvae on sprayed leaves with EO-NLCs, control NLC and treated control with a commercial synthetic insecticide. Bars with different letters indicate significant differences at $p < 0.05$ (ANOVA, LSD post hoc test)*

The tested substances ingested by *S. littoralis* larvae significantly influenced the pest survival in comparison to the untreated control ($F_{5,30}$=8.091; p<0.001) (Fig.5.17A). Similarly, the feeding activity of *S. littoralis* was significantly affected by the EO-NLCs ($F_{5, 30}$=18.36; p<0.0001) (Fig.5.17B). Both Lavender and Rosemary NLC-EOs significantly reduced the ingested leaf area by larvae similarly to the treated control (i.e., lambda cyhalothrin). Interestingly, P-NLC almost doubled the feeding activity of larvae (≈100mm^2) in comparison to the untreated control and the CT-NLC.

Topical exposure



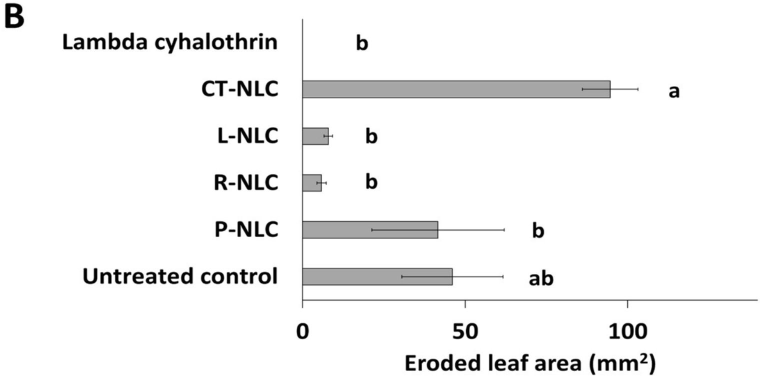


***Figure 5.18**. Mean larval survival (±SE) (A) and mean eroded leaf area (mm^2) (±SE) (B) of* Spodoptera littoralis *larvae sprayed topically with EO-NLCs, control NLC and treated control with a commercial synthetic insecticide. Bars with different letters indicate significant differences at p < 0.05 (ANOVA, LSD post hoc test)*

The tested substances topically sprayed on *S. littoralis* larvae significantly influenced the pest survival ($F_{5,30}$=5.212; *p*=0.00223) (Fig.5.18A) in comparison to the untreated control. Also, the eroded leaf area by *S. littoralis* after topical exposure to EO-NLCs was significantly influenced by the EO-NLC treatment ($F_{5,30}$=10.62; *p* <0.0001). As recorded for P-NLC in the previous bioassay, the CT-NLC increased *S. littoralis* larvae feeding activity (≈90 mm^2) (Fig.5.18B). Only *S. littoralis* larvae topically sprayed with the lambda cyhalothrin (i.e, treated control), as expected, showed neither survival nor feeding activity, resulting both the observed parameters equal to 0%.

The reduction in *S. littoralis* feeding activity recorded in both the tested exposure routes (i.e., ingestion and topical) for Lavender and Rosemary NLCs might be due to the intrinsic toxicity of EOs as previously demonstrated on *Spodoptera* spp. (Ortiz de Elguea Culebras et al., 2018; Sombra et al., 2020). Singular and/or combined EO components, among terpenes and monoterpenes, play a significant role in pest control (Regnault-Roger et al., 2012).

Tuta absoluta

Ingestion exposure

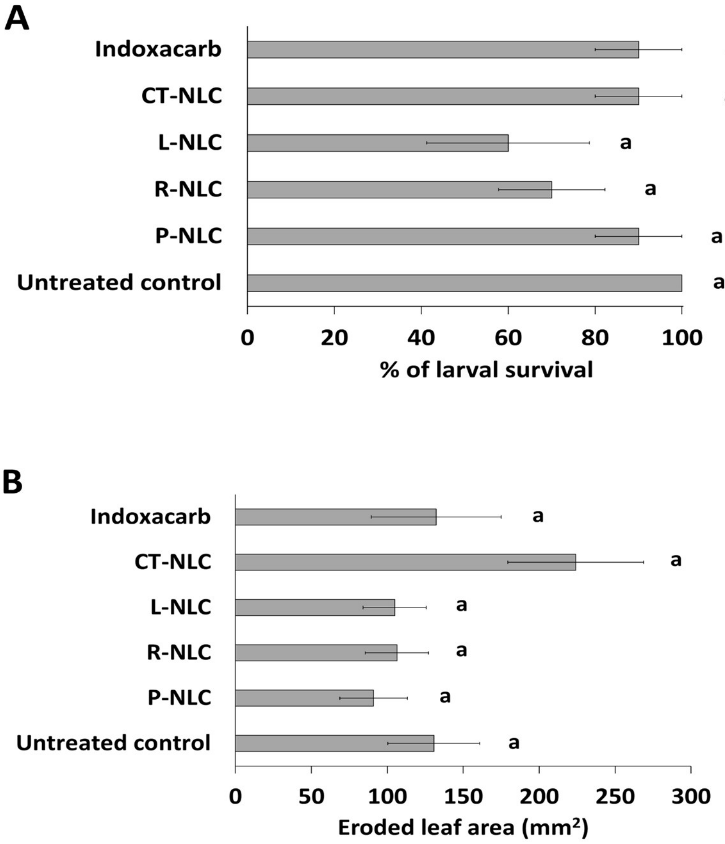
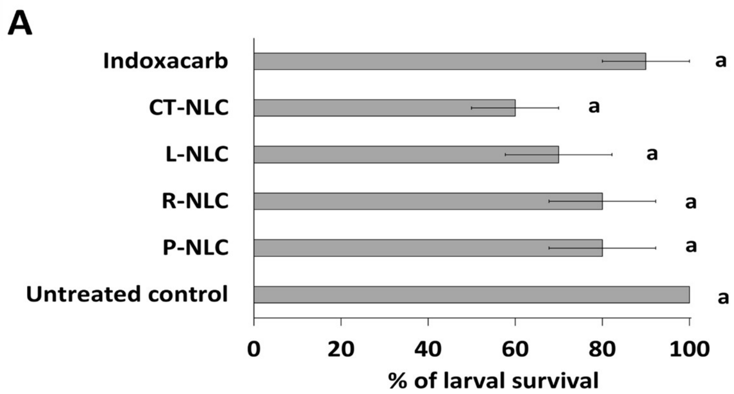


Figure 5.19. *Mean larval survival (±SE) (A) and mean eroded leaf area (mm^2) (±SE) (B) of* Tuta absoluta *larvae on sprayed leaves with EO-NLCs, control NLC and treated control with a commercial synthetic insecticide. Bars with different letters indicate significant differences at* $p < 0.05$ *(ANOVA, LSD post hoc test)*

NLCs with Rosemary, Lavender and Peppermint EOs had no influence on the survival of *T. absoluta* larvae by ingestion exposure ($F_{5,30}$= 1.7; *p*= 0.173) (Fig.5.19A). Also, the measured eroded leaf area by *T. absoluta* larvae after leaf exposure to EO-NLCs was not significantly influenced by the EO-NLC treatment ($F_{5,30}$=2.273; *p*= 0.0795) (Fig.5.19B). However, our observations suggested that CT-NLC may increase the feeding activity of larvae nearly 2-folds more than the untreated control.

Topical exposure



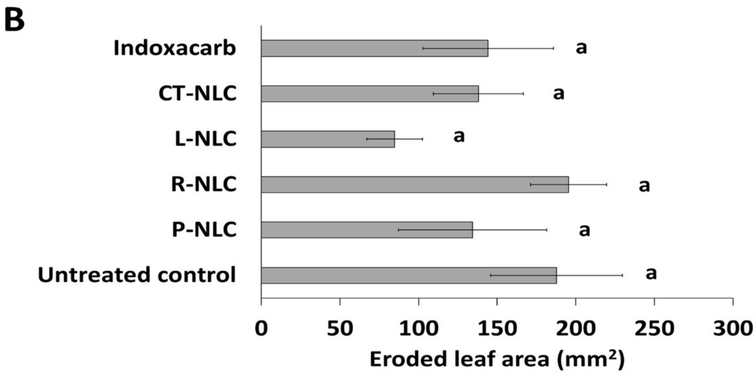


Figure 5.20. *Mean larval survival (±SE) (A) and mean eroded leaf area (mm^2)(±SE) (B) of* Tuta absoluta *larvae sprayed topically with EO-NLCs, control NLC and treated control with a commercial synthetic insecticide. Bars with different letters indicate significant differences at p < 0.05 (ANOVA, LSD post hoc test)*

NLCs with Rosemary, Lavender and Peppermint EOs by topical exposure did not affect the survival of *T. absoluta* larvae ($F_{5,30}$=1.846; *p*= 0.142) (Fig.5.20A). However, L-NLC and the CT-NLC reduced the larval survival up to the ≈30% and ≈40%, respectively. Also, the eroded leaf area by *T. absoluta* after topical exposure to EO-NLCs was not significantly influenced by the EO-NLC treatment ($F_{5,30}$=1.314; *p*= 0.291) (Fig.5.20B). Nonetheless, Lavender EO severely reduced the feeding activity of *T. absoluta*.

Neither acute toxicity nor a reduction in the feeding activity was caused by EO-NLCs against *T. absoluta* in both the exposure routes (i.e., ingestion and topical). The low mortality and the high eroded leaf area observed in *T. absoluta* larvae after ingestion exposure might be explained by the reduced capacity of EO-NLCs to penetrate plant foliar tissue and by *T. absoluta* feeding strategy (Campolo et al., 2017). After hatching, young larvae penetrate leaves, stems, or fruits in which they feed and develop, avoiding the direct exposure to the EO-NLCs. Moreover, the low topical toxicity and the high feeding activity after EO-NLCs treatment on the leaf, might be due to the scarce capacity of EO-NLCs to penetrate the insect body and the potential combined ability of larvae to detoxify the nanocarried EOs (Ghanim & Abdel Ghani, 2014).

Although NLC technology has been used for encapsulating synthetic insecticides (Maroofpour et al. 2021), no information is currently available on the use of this nanotechnology in EO delivery for pest control. To the best of our knowledge, this is the first attempt in using such a promising technology in applying EOs for controlling three economic relevant insect pests having different feeding strategies.

Conclusions

Our results have demonstrated that EOs nanoencapsulation allowed obtaining stable colloidal formulations characterized by homogeneous and small particles. The developed EO-NLCs demonstrated to be able to provide plant protection as alternative sustainable tools and reduce the negative environmental impact of conventional management systems. The EO-NLCs were evaluated for stability and miscibility and the results are promising, mostly regarding R-NLC and CT-NLC in terms of long-term stability. For homogeneity the following stability scale could be defined: R-NLC > L-NLC > P-NLC > CT-NLC.

In summary, for *A. gossypii* all the tested EO-NLCs caused high mortality and significantly reduced its progeny by topical exposure. Similarly, for *S. littoralis*, Lavender and Rosemary EO-NLCs decreased the feeding activity but not the survival. Conversely, for *T. absoluta* Lavender, Peppermint and Rosemary EO-NLCs did not decrease the survival and the feeding activity. Therefore, to obtain encouraging results it is probably necessary to increase the v/v of EOs in NLCs.

Our findings suggest that NLCs can successfully deliver EOs and keep their properties unaltered without penetrating plant tissues, as suggested by the results observed on *T. absoluta*. Nevertheless, the outcomes of the present work need to be supported by further studies on EOs and NLCs, focusing on their delivery dynamics within plant and insect tissue (Athanassiou et al., 2018; Karny et al., 2018). Moreover, the bioactivity of these EOs need to be explored thoroughly also towards non-target organisms for evaluating the environmental risk assessment of these potential bio-rational insecticides (Campolo et al., 2020b; Pavela et al., 2020).

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6 Appendix B: Contribution to Congress proceedings

14th meeting of the IOBC-WPRS Working Group "Integrated Control in Protected Crops, Mediterranean Climate", 4-7 September 2018, Lisbon (Portugal)

Plant suitability to the South American tomato pinworm and pest-induced resistance in 11 tomato varieties

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A large number of arthropod species can infest tomato plants. Among key tomato pests, the South American tomato pinworm, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), is one of the most dangerous, particularly across the Afro-Eurasian continent, since its first invasion in Europe in 2006 (Campos et al., 2017). It causes drastic tomato yield losses owing to its leaf-mining activity and through fruit infestations (Biondi et al., 2018). *Tuta absoluta* has been historically subjected to chemical control since the seventies in South America, however this strategy led to the emergence of multi-resistant populations (Guedes and Siqueira, 2013). As a consequence, alternative control strategies have to be prioritized and, among them, the use of resistant varieties could represent a valid sustainable control strategy. However, despite the multiple constitutive and induced processes that can mediate tomato plant defenses against pests in wild and cultivated tomatoes (Naselli et al., 2016; Vargas-Ortiz et al., 2018), the commercial development of *T. absoluta*–resistant tomato cultivars is in progress.

Within this context, we aimed at assessing the suitability of leaves cut from several tomato varieties as feeding substrate for larvae of *T. absoluta*. Eleven tomato varieties were chosen among those commercially grown in protected crops of the Southern Mediterranean area on the bases of different agronomic (e. g., seasonality of the cycle) and fruit type (e. g., Marmande, Cherry, Date, etc.) features. The tested varieties were 'Better Bush', 'Cikito', 'Delizia', 'Faustyno', 'Marinda', 'Motekino', 'Pixel', 'Rovente', 'Shiren', 'SV1201' and 'Tyty'. Then, we assessed the potential for *T. absoluta* larvae to elicit systemic defense mechanisms in these varieties. For this, we used healthy leaves cut from tomato plants previously infested with *T. absoluta* larvae to let developing pest larvae.

Results showed a strong diversity in plant suitability for the development of *T. absoluta* larvae in terms of larval survival, larval development time, weight of the pupae and consumed leaf area. Similarly, the results suggest a that few varieties have potential for induced plant defense, because on these varieties *T. absoluta* larvae developing on leaves cut from pre-infested plants suffered a higher mortality and fed less than those feeding on leaves cut from healthy plants. Such results suggest a possible direct antibiosis and antifeedant activity of induced allelochemicals on *T. absoluta* larvae.

These findings represent the first bases for selecting resistant/tolerant tomato varieties in the Mediterranean protected tomato environment. However, further studies will be performed to detect the chemicals and metabolic biochemical processes behind this phenomenon, in order to understand whether the induced resistance found in certain varieties is also consistent at the plant volatile level, i. e., toward flying adult females of *T. absoluta*. Moreover, studies at the multi-trophic level, i. e., including *T. absoluta* natural enemies, should be performed prior promoting the more tolerant varieties into integrated pest management programs.

Keywords: Allelochemical, Chemical ecology, IPM, Invasive pest, Plant defense, *Tuta absoluta*

Acknowledgements

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Plant suitability to the South American tomato pinworm and pest-induced resistance in wild and commercial *Solanum* species

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Among key tomato pests, the South American tomato pinworm, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), is one of the most dangerous, particularly across the Afro-Eurasian continent since its first invasion in Europe in 2006. This pest has been historically subjected to chemical control since the seventies in South America, however this strategy led to the emergence of multi-resistant populations. As a consequence, alternative control strategies have to be prioritized and, among them, the exploitation of plant resistance could represent a valid sustainable control tool. Within this context, eleven tomato varieties were chosen among those commercially grown in protected crops of the Southern Mediterranean area on the bases of different agronomic traits and fruit types. The suitability of leaves from healthy plants of the varieties as feeding substrate for larvae of *T. absoluta* was estimated. Results showed a strong diversity in plant suitability for the development of *T. absoluta* larvae in terms of larval survival, larval development time, weight of the pupae and eroded leaf area. The potential for *T. absoluta* larvae to elicit systemic resistance mechanisms was also assessed. For this, we used healthy leaves from plants previously infested with *T. absoluta* larvae. The

obtained results suggested that few varieties have potential for induced plant resistance, because on these species *T. absoluta* larvae developing on leaves from pre-infested plants suffered a low survival and fed less than those feeding on leaves from healthy plants. Therefore, the varieties showing promising results, together with wild and further commercial species belonging to the *Solanum* genus (e.g., eggplant), are going to be test in further experiments. These are aiming at assessing the susceptibility to the pest attack, the potential pest-induced resistance mediated by resistance genes expressing the synthesis of allelochemicals, both volatile and non-volatile compounds. The results will provide evidences on potential direct antibiosis and antifeedant activities of induced substances on *T. absoluta* behavior and development.

Plant resistance induced by the South American tomato pinworm in wild and commercial *Solanum* species

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Plant-insect interactions have been for a long time studied in both general and applied science, e.g., for optimized Integrated Pest Management. The South American tomato pinworm, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), is a very harmful pest of tomato that can develop on several solanaceous species. This insect has been historically subjected to chemical control and, owing to pesticide side effects, alternative control strategies, such as resistant host plants, must be prioritized. Within this context, we assessed the biochemical consequences of *T. absoluta* infestation and the subsequent plant suitability for the pest development in several *Solanum* species and tomato varieties. The accumulation of secondary metabolites, the main enzymatic activities and the emission of organic volatile compounds were identified for both control and *T. absoluta*-induced plants. The host species showing promising results in preliminary experiments and in a literature search, together with wild and further commercial species (e.g., eggplant) grown under protected conditions in the south Mediterranean area were used in biological and biochemical experiments. The results suggest that some *Solanum* species and tomato varieties have potential for induced plant resistance via direct antibiosis and antifeedant activities. These findings represent the first bases for selecting resistant/tolerant tomato varieties under the Mediterranean protected tomato growing conditions.

Keywords
IPM, Plant defense mechanism, Pest resistance, Secondary metabolites, *Tuta absoluta*

Herbivore induced VOCs, secondary metabolites and enzymatic activity in *Solanum* species

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Tuta absoluta (Meyrick) (Lepidoptera: Gelechiidae) is one of the most dangerous pests of tomato that can also develop on several other solanaceous species. This insect has been traditionally subjected to chemical control and alternative control strategies, must be prioritized. Among these, the exploitation of the plant-insect interactions and plant resistance could represent sustainable control option for optimizing Integrated Pest Management. Within this context, e biochemical consequences of *T. absoluta* infestation and the subsequent plant suitability for the pest development in several *Solanum* species and tomato varieties were assessed. Healthy leaves from plants previously infested with *T. absoluta* larvae (induced plants) have been used and the obtained results suggested that few species and varieties, such as the eggplant ‘Black beauty’, have potential for induced plant resistance. In induced plants *T. absoluta* larvae suffered a low survival and fed less than those feeding on leaves from healthy plants. Then, s responsible for the defense interaction between *T. absoluta* and the tested plants were characterized by identifying the secondary meabolites, such as polyphenols, the enzymatic activities (i.e., PPO and POD), and the organic volatile compounds, e.g., terpenes. The results provide evidences of potential direct antibiosis and antifeedant activities of induced substances on *T. absoluta* behavior and development. These findings represent the first bases for selecting resistant/tolerant tomato varieties.

Herbivore induced VOCs, secondary metabolites and enzymatic activity in *Solanum* spp.

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Plants are able to respond to abiotic and biotic stresses from the external environment, including insect herbivores, with complex defense responses which involve resistance and adaptation to survive. Metabolites, mechanical barriers, enzymes and volatile compounds constitute the biochemical and physical, constitutive and induced defense mechanisms of the plants. In this framework, 12 species/varieties of *Solanum* spp. were tested in order to evaluate the plant defense response to the infestation by the tomato key pest *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). In particular, induced secondary metabolites, such as polyphenols, enzymes involved in oxidation processes (i.e., PPO and POD), and volatile compounds, e.g. terpenes, were identified and characterized. In addition, bio-assays on larval survival, larval development time, pupal weight and eroded leaf area were assessed using cut leaves from healthy plants and from plants previously infested with *T. absoluta* larvae (induced plants). In

most of the tested plants, and especially in the tested eggplant variety, had an effect on *T. absoluta* larval survival and feeding activity. Furthermore, many compounds were identified among induced secondary metabolites, especially in wild species, *Solanum pennelli* and *Solanum habrochaites*. Some of the cultivated varieties showed an array of induced volatiles very likely implied in plant communication mechanisms related to their interaction with pests and/or natural enemies. The results suggested that these species/varieties have potential for induced plant resistance against this pest, potential direct antibiosis and antifeedant activities of induced substances on *T. absoluta* behavior and development is evidenced.

Herbivore induced VOCs, secondary metabolites and enzymatic activity in *Solanum* spp.

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had an effect on *T. absoluta* larval survival and feeding activity. Furthermore, many compounds were identified among induced secondary metabolites, especially in wild species, *Solanum pennelli* and *Solanum habrochaites*. Some of the cultivated varieties showed an array of induced volatiles very likely implied in plant communication mechanisms related to their interaction with pests and/or natural enemies. The results suggested that these species/varieties have potential for induced plant resistance against this pest, potential direct antibiosis and antifeedant activities of induced substances on *T. absoluta* behavior and development is evidenced.

Plant resistance induced by the South American tomato pinworm in wild and commercial *Solanum* species

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Plant-insect interactions have been studied for a long time in both general and applied science, e.g., for optimized Integrated Pest Management. The South American tomato pinworm, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), is a key pest of tomato that can although develop on several solanaceous species. This insect has been traditionally subjected to chemical control and, owing to pesticide side effects (e.g. insecticide resistance), alternative control strategies, such as resistant host plants, must be prioritized. Within this context, we assessed the biochemical consequences of *T. absoluta* infestation and the subsequent plant suitability for the pest development in several *Solanum* species and tomato varieties. The accumulation of secondary

metabolites, the main enzymatic activities and the emission of organic volatile compounds were identified for both control and *T. absoluta*-induced plants. The host species showing promising results in preliminary experiments and in a literature search, together with wild and further commercial species (e.g., eggplant *Solanum melongena*) grown under protected conditions in the south Mediterranean area were used in biological and biochemical experiments. The results suggest that some *Solanum* species and tomato varieties have potential for induced plant resistance via direct antibiosis and antifeedant activities. These findings represent the first bases for selecting resistant/tolerant tomato varieties under the Mediterranean protected tomato growing conditions.

Keywords

IPM, Plant defense mechanism, Pest resistance, Secondary metabolites, *Tuta absoluta*

Multilevel analyses of pest induced defenses in solanaceous plants

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Plants are able to respond to abiotic and biotic stresses, including insect herbivores, with complex defense responses. Defense mechanisms in cultivated plants represent an important tool for their integrated pest control packages. In particular, plant volatiles (VOCs) and induced volatiles (HIPVs) can play a key role in indirect plant defense, involving multi-trophic levels. VOCs and HIPVs are important to allow plant-plant and plant-natural enemy communications. In this context, I conducted olfactory bioassays, I identified VOCs and HIPVs, and I evaluated the gene expression of tomato and aubergine plants primed by *Tuta absoluta* (Lepidoptera: Gelechiidae) larvae. In the tests, for tomato var ‘Delizia’, var ‘Cikito’, var ‘Marinda’, var ‘Optima’, var ‘Rovente’, var ‘San Marzano nano’' and var ‘Tyty’ were used and for aubergine var. ‘Black beauty’. Adults of *T. absoluta* and *Encarsia formosa* (Hymenoptera: Aphelinidae) were used in olfactory bioassays to understand how the key tomato pest and a model parasitoid are attracted to VOCs and HIPVs emitted by primed plants. The evaluation of the gene expression of three genes were done to highlight defense plant pathways, i.e., jasmonic acid, salicylic acid and β-phellandrene. The results showed that in the identification of volatiles there was a significant increasing of production of primed volatiles. This explains the pest and parasitoid olfactory responses that varied among primed and non-primed tomato and aubergine varieties. The results of the gene expression analyses provided an important evidence of defense induction in the plant. These results are useful for understanding plant defense mechanisms and, above all, can provide volatile compounds involved in the defense of plants that could be used in integrated pest control, for example through attractive dispensers for natural enemies, and in multi-trophic relationships.

Multilevel analyses on pest primed and not-primed solanaceous plants

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Complex plant defense responses are involved in biotic and abiotic stresses, including insect herbivores. In particular, plant volatiles (VOCs) and induced volatiles (HIPVs) can play a key role in indirect plant defense, involving multi-trophic levels. VOCs and HIPVs are important to allow plant-plant and plant-natural enemy communications. In this context, olfactory bioassays were conducted, VOCs and HIPVs were identified, and gene expression of tomato and aubergine plants primed by *Tuta absoluta* (Lepidoptera: Gelechiidae) larvae was evaluated. In the tests, seven tomato and one aubergine varieties were used. Adults of *T. absoluta* and *Encarsia formosa* (Hymenoptera: Aphelinidae) were used in olfactory bioassays to understand how the key tomato pest and a model parasitoid are attracted to VOCs and HIPVs emitted by primed plants. The evaluation of the expression of three genes were done to highlight defense plant pathways, i.e., jasmonic acid, salicylic acid and Bphellandrene. The results showed that there was a significant increase in the production of primed volatiles. This explains the pest and parasitoid olfactory responses that varied among primed and not-primed tomato and aubergine varieties. The results of the gene expression analyses provided an important evidence of defense priming in the plant. These results are useful for understanding plant defense mechanisms and, above all, can provide volatile compounds involved in the defense of plants that could be used in integrated pest control, for example through attractive dispensers to enhance the biocontrol services of natural enemies, and in multi-trophic relationships.

Nanostructured lipid carriers with essential oils against *Tuta absoluta*, *Spodoptera littoralis* and *Aphis gossypii* for sustainable pest management

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Plant essential oils (EOs) represent a promising tool for the control of insect pests. Their practical implementation is hindered by various drawbacks, such as stability and degradation patterns, and phytotoxicity. Innovative and advanced nanotechnologies, such as Nanostructured Lipid Carriers (NLCs), could help overcoming such problems. NLCs, composed of 10%w/v of lipid and 10% w/v of oil (lavander, rosemary, mint) were prepared through PIT method. The obtained formulations showed the presence of small (200 nm) and homogeneous particles (PDI < 0.3). All NLCs were purified obtaining a 20% w/v EO concentration using ultracentrifugation, without any significant variation of mean size and homogeneity. The efficacy of these formulations was tested for three key insect pests with different feeding strategies: a leafminer (*Tuta absoluta*), a chewer (*Spodoptera* *littoralis*) and a sap sucking (*Aphis gossypii*). NLCs loaded with EOs were diluted with distilled water at 30% and were tested in two exposure routes, topically and by ingestion. Long-term stability results obtained by Turbiscan® technology showed the occurrence of not significant (ΔBS < 20%) sedimentation phenomena after 30 days of storage at 25°C. Among the three EOs, rosemary demonstrated to be the most long-term stable, as confirmed by the destabilization kinetic. The results of the bioassays showed that the three EO-NLCs did not significantly decrease the survival and the feeding activity of *T. absoluta*. Conversely, the NLCs of lavender and rosemary EOs decreased the feeding activity but not the survival of *S. littoralis*. Interestingly, all the tested EO-NLCs, as well as the nanocarrier alone, caused high mortality on *A. gossypii* and significantly reduced its progeny by topical exposure. Our findings suggest that NLCs can successfully deliver EOs and deserve potential for controlling agricultural pests.

Keywords

Essential oil, Nanotechnology, NLC, Pest control, Sustainability