



SCUOLA di
DOTTORATO

Dottorato in Architettura
Dottorato in Diritto ed Economia
Dottorato in Ingegneria Civile, Ambientale e Industriale
Dottorato in Ingegneria dell'Informazione
Dottorato in Scienze Agrarie, Alimentari e Forestali

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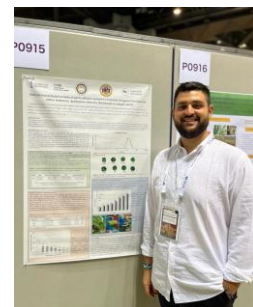
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BIOLOGICAL ACTIVITY OF GARLIC ESSENTIAL OIL
NANOFORMULATIONS FOR THE MANAGEMENT OF
VEGETABLE PESTS
PH.D. THESIS

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Abstract

From an economic point of view, the horticultural sector has a significant impact globally, in fact, according to FAOSTAT data of 2021, the value of horticultural production is around USD 1.1 trillion per year with a production of about 1.1 billion tons.

Despite its economic potential, the horticultural sector faces numerous challenges, including price volatility due to climatic factors, pests and diseases.

Increasing temperatures, irregular rainfall and extreme weather events are changing the growing conditions of crops, altering growth cycles, affecting yields and favoring the spread of pests and diseases.

Pests and plant diseases are a major threat to horticultural production, and their impact can lead to crop losses of 20-50% or even 100%, depending on the type of pest and level of infestation. Even the slightest infestation can compromise the visual and nutritional quality of the products, making them less attractive to consumers and lowering the market price. Pest management is a critical aspect of modern agriculture, requiring substantial investments in pesticides, monitoring technologies, and agronomic interventions to ensure crop health and productivity.

The indiscriminate and intensive use of synthetic insecticides entails environmental risks, such as soil and water pollution, risks to human health and to non-target insects.

The control of phytophagous insects is also particularly complex due to the limitations on the use of synthetic chemical insecticides and the development of resistance to the few active ingredients currently allowed, which render chemical treatments ineffective. In fact, over the last 30 years, the world of chemical insecticides globally has undergone radical changes both in terms of the types of

substances used and in terms of regulations leading to the introduction of sustainable management practices.

Another important factor related to the use of insecticides for pest control is the growing consumer demand for pesticide-free food, driven by an increased awareness of health and environmental risks. Among the alternatives to the use of synthetic insecticides, botanical extracts, in particular essential oils, are receiving considerable interest. Essential oils are important secondary metabolites of plants, which, when properly formulated, contribute to the control of phytophagous insects and are also low or non-toxic to non-target organisms, safe for humans, the environment and the ecosystem.

The main objective of this thesis was to develop different nanoformulations based on garlic essential oil, using high energy and different pressures, such as 2000, 20.000 and 30.000 psi. Garlic nanoemulsions were tested against three key pests of horticultural crops, such as *Spodoptera littoralis*, *Phthorimaea (Tuta) absoluta* and *Bemisia tabaci*, with the aim of assessing their insecticidal potential. In addition, adverse effects on non-target organisms such as the generalist predator *Nesidiocoris tenuis* and possible phytotoxic effects on plants such as tomato, pepper, cucumber and eggplant were also evaluated.

The results obtained from this research are promising, as the garlic nanoformulations had an interesting insecticidal effect against the three chosen target insects. Furthermore, the developed nanoemulsions were low toxic towards the mirid predator and only the highest doses were somewhat phytotoxic towards cucumber and eggplant.

Therefore, based on the results obtained, we can state that the developed and tested garlic nanoemulsions could be a viable alternative to the use of chemical insecticides for phytophagous control, although further studies on natural enemies are needed.

Riassunto

Da un punto di vista economico, il settore orticolo incide notevolmente a livello globale, infatti secondo i dati FAOSTAT del 2021, il valore della produzione orticola si aggira intorno ai 1.1 trilioni di dollari l'anno con una produzione di circa 1.1 bilioni di tonnellate.

Nonostante il potenziale economico, il settore orticolo deve affrontare una serie di sfide, tra le quali la volatilità dei prezzi, dovuta a fattori climatici, parassiti e malattie.

L'aumento delle temperature, le precipitazioni irregolari e gli eventi meteorologici estremi, stanno modificando le condizioni di crescita delle colture, causando un'alterazione dei cicli di crescita, influenzando la resa e favorendo la diffusione di parassiti e malattie.

I parassiti e le malattie delle piante rappresentano una delle principali minacce per la produzione orticola, infatti la loro presenza può portare a perdite di raccolto del 20-50% o anche del 100%, a seconda del tipo di parassita e del livello di infestazione. Anche una minima infestazione può compromettere la qualità visiva e nutrizionale del prodotto, rendendolo meno attraente per i consumatori e abbassandone il prezzo di mercato.

La gestione dei parassiti richiede investimenti significativi in termini di pesticidi, tecnologie di monitoraggio e interventi agronomici.

L'uso indiscriminato e intensivo di insetticidi di origine sintetica comporta rischi ambientali, come l'inquinamento del suolo e dell'acqua, rischi per la salute umana e per gli insetti non bersaglio.

Il controllo degli insetti fitofagi è particolarmente complesso anche a causa delle limitazioni all'uso degli insetticidi chimici di sintesi e dello sviluppo di resistenze ai pochi principi attivi attualmente ammessi, che rendono inefficaci i trattamenti chimici. Infatti, negli ultimi 30 anni, il mondo degli insetticidi chimici a livello globale ha subito cambiamenti radicali sia in termini di tipologie di

sostanze utilizzate, sia in termini di normative che portano all'introduzione di pratiche di gestione sostenibile.

Altro fattore importante, legato all'uso degli insetticidi per il controllo dei fitofagi, riguarda la sempre più crescente domanda dei consumatori per cibo senza pesticidi, spinta da una maggiore consapevolezza sui rischi per la salute e l'ambiente.

Tra le alternative all'uso degli insetticidi di sintesi, gli estratti botanici, in particolare gli oli essenziali, stanno riscontrando notevole interesse. Gli oli essenziali sono importanti metaboliti secondari delle piante, che, se opportunamente formulati, contribuiscono al controllo degli insetti fitofagi, risultando inoltre poco o non tossici per organismi non bersaglio, sicuri per l'uomo, per l'ambiente e per l'ecosistema.

L'obiettivo principale di questa tesi è stato quello di sviluppare differenti nanoformulazioni di olio essenziale d'aglio, utilizzando alta energia e differenti pressioni, come 2000, 20000 e 30000 psi. Queste nanoemulsioni d'aglio, sono state testate contro tre importanti fitofagi delle colture orticole, come *Spodoptera littoralis*, *Phthorimaea (Tuta) absoluta* e *Bemisia tabaci*, con l'obiettivo di valutare il potenziale insetticida. Inoltre, sono stati valutati gli effetti negativi su organismi non bersaglio, come il predatore generalista *Nesidiocoris tenuis* e gli eventuali effetti fitotossici nei confronti di piante come pomodoro, peperone, zucchini e melanzana.

I risultati ottenuti di questa ricerca sono promettenti, in quanto le nanoformulazioni d'aglio hanno avuto un'efficacia insetticida nei confronti dei tre insetti target scelti. Inoltre, sono risultate poco tossiche nei confronti del predatore e solo le dosi più alte sono risultate leggermente fitotossiche nei confronti di zucchini e melanzana.

Quindi, in base ai risultati ottenuti, possiamo affermare che le nanoemulsioni d'aglio sviluppate e testate, potrebbero essere una valida alternativa all'uso di insetticidi chimici per il controllo dei fitofagi, anche se sono necessari ulteriori studi sui nemici naturali.



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Keywords. Botanical extract; Bioinsecticide; Lepidoptera; Whitefly; Leaf miner; Horticultural sector; Biocontrol



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I. Introduction

I.I. Importance of vegetable crops in the world and in Italy

According to data from the Food and Agriculture Organization (FAO), in 2021 the world's vegetable production exceeded 1.1 billion tons. The Countries with the highest volume of vegetable production are China, India, the USA, Turkey and Italy. China alone contributes more than 50 per cent of the world's vegetable production, with crops such as garlic, cabbage and tomatoes as the most cultivated ones. India is the second largest producer, with a focus on onions, tomatoes and eggplants.

From an economic point of view, the global vegetable market generates a huge volume of business: the global turnover of the vegetable sector is estimated at around \$1.1 trillion (2021). This includes production for the fresh market as well as for industrial processing (juices, canned and frozen products). The economic value of some crops is particularly high due to their versatility and wide use in many global cooked food products; for example, tomatoes generate over \$190 billion in global annual turnover (FAOSTAT, 2022).

Horticulture in Italy represents an important sector of agricultural production. In fact, about 14% of agricultural production is made up of the horticultural sector (Bertoni and Cavicchioli, 2016), with a turnover of about EUR 7.3 billion per year. Approximately 300,000 hectares are used for the cultivation of horticultural crops, 11% of which are grown in greenhouses. The products are sold as fresh products, processed and IV gamma products (Bertoni and Cavicchioli, 2016).

Italy is the 2nd largest vegetable producer in Europe after Spain (FAOSTAT, 2022), but it is first in Europe for tomato and eggplant production. The turnover of the Italian horticultural sector is noteworthy, contributing significantly to the national agricultural economy. During the 2021, the turnover of horticultural crops in Italy was around EUR 12-13 billion (ISTAT, 2021). The crop with

the greatest economic impact is the processing tomato, used for preserves and sauces, which generates an annual turnover of around 3 billion of euro (ANICAV, 2021).

Vegetable crops such as tomato, eggplant, pepper and cucumber, (i.e. crops closely related to this Ph.D thesis), are among the most cultivated crops in Italy and occupy an area of 97,610, 9,600, 9,390 and 1,980 ha respectively. According to the latest FAOSTAT (2022) data, the production of these vegetables is the following: 6,136,380 t of tomato, 307,430 t of eggplant, 232,680 t of pepper and 61,830 t of cucumber.

Among the aforementioned crops, tomato represents the vegetable crop to have the greatest economic impact on the whole horticultural sector (FAOSTAT, 2022). Considering both imports and exports, Italy's tomato industry boasts a turnover exceeding 4 billion euros, making the country the third-largest tomato processor globally after the USA and China, and the second-largest exporter in the world (ISMEA, 2022). The Italian regions most actively involved in vegetable production are Sicily, Campania, Apulia, and Veneto. Despite its important economic contribution, the vegetable sector faces several challenges: climate change, and extreme weather events, such as droughts and floods, are negatively affecting production. In addition, the increasing spread of new pests and diseases, such as *Phthorimaea (Tuta) absoluta* (Lepidoptera: Gelechiidae), is threatening the yield of vegetable crops.

The environmental impact of tomato production is significant, primarily due to water usage, pesticide application, and soil degradation. In regions located in southern Italy, water scarcity is a growing concern. Innovative solutions like precision irrigation, drip systems, and rainwater harvesting are needed to reduce water waste. Moreover, the overuse of chemical fertilizers and pesticides has led to soil depletion and biodiversity loss, requiring a shift towards organic farming methods, integrated pest management, and the use of biofertilizers. The future of tomato production hinges on the integration of innovation and sustainability. By adopting water-saving technologies, reducing energy

consumption, developing climate-resilient crops, and implementing circular economy principles, the industry can become more sustainable while maintaining high productivity levels. This shift will not only address environmental concerns but also improve economic resilience and market competitiveness. (CIA-AGRICOLTORI ITALIANI, 2021).

I.II. Pests of vegetable crops

Horticultural crops are a cornerstone of Italy's agricultural sector, contributing significantly to both domestic consumption and exports. However, the productivity and quality of these crops are constantly threatened by a wide range of pests. These pests not only affect yields but also increase production costs, reduce marketability, and pose challenges for farmers who strive to produce high-quality vegetables sustainably. In recent years, the increasing pressure of climate change, along with the rise of pest resistance to conventional pesticides, has heightened the need for more effective and environmentally friendly pest management strategies.

Understanding the most common pests affecting Italy's vegetable crops, as well as the methods available to mitigate their impact, is essential for ensuring the long-term sustainability and profitability of the sector.

Horticultural plants are attacked by many phytophagous species belonging to different taxa. Moreover, these pests also differ in the type of damage attacking the roots, leaves, flowers, fruits or the whole plant, depending on the mouthpart they possess. Sucking insects, such as aphids and whiteflies, not only pierce and feed on plant saps but also serve as vectors for various viruses. Aphids, in particular, can transmit diseases like Cucumber mosaic virus (CMV) and Potato Y virus (PVY),

while whiteflies are known to spread Tomato infectious chlorosis virus (TICV) and Tomato chlorosis virus (ToCV) (CNR, 2024). On the other hand, chewing insects, such as lepidopteran larvae, cause damage primarily feeding on leaves, fruits, and even entire plants. Pests cause direct damage to plants by feeding on various plant tissues, but they also inflict indirect harm. Through the type of damage they cause, insects can act as vectors for diseases, such as viruses, and create entry points for secondary pathogens like fungi and bacteria. These secondary infections further degrade the quality and appearance of the crop, often rendering the fruit unsaleable. This combined damage can significantly impact on the agricultural sector, leading to economic losses and reduced productivity. Another important aspect to consider with horticultural pests is their pronounced polyphagy, meaning they often do not have a preferred host but can adapt to the available horticultural crop at any given time. However, there are exceptions, as in the case of *P. absoluta*, which is known for its almost exclusive oligophagy, primarily targeting tomato plants. This characteristic found in most insect pests makes them a greater threat to diverse vegetable crops, as they can switch hosts based on availability. Moreover, with the introduction of new, intensive, high-yielding hybrids and varieties, along with the changing climate of the ecosystem and the adaptability of insects to this, the horticultural sector is facing new and increasingly tough challenges. These factors have contributed to significant losses, with up to 40% of vegetable production being affected by insect infestations (Maish SC, 2019). Production losses caused by insects translate into a considerable economic impact for farmers and entire agricultural sectors. In some cases, farmers have to bear additional costs for the purchase of pesticides and the adoption of control techniques, reducing profit margins. Direct and indirect losses caused by insects can cause economic crises, especially in developing regions where agriculture is the main source of income.

Agricultural production losses due to insects are a significant global problem, with important economic, environmental and social impacts. According to FAO estimates, losses due to insects and

other biological agents can reach up to 20-40% of global agricultural production each year. In some regions and for specific crops, these losses can be even higher (FAO, 2022).

Vegetable crops in Italy, including tomatoes, face significant challenges from various pests that can damage yields, affect quality, and increase production costs. Some of the most common and economically important pests in Italian agriculture include the following species (Table 1):

Order	Family	Genus	Main Key-Pest	Main Vegetable Crops	Damaged Organs
Hemiptera	Aphididae	Aphis	<i>Aphis gossypii</i>	Cucurbitaceae; Solanaceae; Brassicaceae; Malvaceae; Fabaceae	Leaves, flowers and fruits
		Aphis	<i>Aphis fabae</i>		
		Macrosiphum	<i>Macrosiphum euphorbiae</i>		
		Myzus	<i>Myzus persicae</i>		
	Aleyrodidae	Bemisia	<i>Bemisia tabaci</i>	Cucurbitaceae; Solanaceae; Malvaceae	Leaves
		Trialeurodes	<i>Trialeurodes vaporariorum</i>	Cucurbitaceae; Solanaceae; Malvaceae	Leaves
Miridae	Nesidiocoris	<i>Nesidiocoris tenuis</i>	Solanaceae; Cucurbitaceae	Vegetative tips	
Pseudococcidae	Phenacoccus	<i>Phenacoccus solenopsis</i>	Cucurbitaceae; Solanaceae; Malvaceae	Vegetative tips, leaves, stems and fruits	
Lepidoptera	Noctuidae	Spodoptera	<i>Spodoptera littoralis</i>	Cucurbitaceae; Solanaceae; Brassicaceae; Malvaceae; Fabaceae; Asteraceae	Leaves and fruits
		Spodoptera	<i>Spodoptera exigua</i>		
		Helicoverpa	<i>Helicoverpa armigera</i>		
		Autographa	<i>Autographa gamma</i>		
		Chrysodeixis	<i>Chrysodeixis chaldites</i>		
		Mamestra	<i>Mamestra brassicae</i>		
		Agrotis	<i>Agrotis spp.</i>		
	Gelechiidae	Phthorimaea	<i>Phthorimaea absoluta</i>	Solanaceae	Leaves, stems and fruits
			<i>Phthorimaea operculella</i>		Tuber
Crambidae	Ostrinia	<i>Ostrinia nubilalis</i>	Poaceae	Leaves, stems and fruits	
Rhynchota	Pentatomidae	Nezara	<i>Nezara viridula</i>	Cucurbitaceae; Solanaceae; Brassicaceae; Malvaceae; Asteraceae	Leaves and fruits
		Halyomorpha	<i>Halyomorpha halys</i>		
Coleoptera	Curculionidae	Otiorrhynchus	<i>Otiorrhynchus spp.</i>	Rosaceae	Roots and leaves
	Chrysomelidae	Leptinotarsa	<i>Leptinotarsa decemlineata</i>	Solanaceae	Leaves, flowers and fruits
Acarina	Tetranychidae	Tetranychus	<i>Tetranychus urticae</i>	Cucurbitaceae; Solanaceae; Malvaceae; Rosaceae; Poaceae	Stems, leaves, flowers and fruits
	Tarsonemidae	Polyphagotarsonemus	<i>Polyphagotarsonemus latus</i>	Solanaceae; Cucurbitaceae; Malvaceae	
	Eriophyidae	Aculops	<i>Aculops lycopersici</i>	Solanaceae	
Thysanoptera	Thripidae	Frankliniella	<i>Frankliniella occidentalis</i>	Cucurbitaceae; Solanaceae; Brassicaceae; Malvaceae; Fabaceae; Liliaceae	Buds, leaves, flowers and fruits
Diptera	Agromyzidae	Liriomyza	<i>Liriomyza spp.</i>	Cucurbitaceae; Solanaceae; Brassicaceae; Malvaceae; Fabaceae; Liliaceae; Asteraceae	Leaves
Tylenchida	Heteroderidae	Meloidogyne	<i>Meloidogyne spp.</i>	Cucurbitaceae; Solanaceae; Brassicaceae; Malvaceae; Fabaceae; Asteraceae	Roots
Orthoptera	Gryllotalpidae	Gryllotalpa	<i>Gryllotalpa gryllotalpa</i>	Cucurbitaceae; Solanaceae; Brassicaceae; Malvaceae; Fabaceae; Asteraceae	Roots

Table 1. Main key phytophagous insects of horticultural crops and the damage they cause to crops.

*I.II.I. Biology, ecology and management of *Spodoptera littoralis*, *Bemisia tabaci*, *Phthorimaea absoluta**

Pests infesting horticultural crops are a significant concern as they can lead to reduced yields, degraded quality, and economic losses. These pests range from insects to nematodes, fungi, and bacteria, each causing specific damage to plants. Chewing insects, such as caterpillars and beetles, directly feed on leaves, stems, and fruits, leaving visible damage and weakening plants. Sap-sucking insects, including aphids, whiteflies, and mealybugs, feed on plant juices, causing wilting, stunted growth, and reduced plant vigor. In addition, they can transmit viral diseases that further impair crop health.

*I.II.I.I. *Spodoptera littoralis**

Among the different pests of horticultural crops, some Lepidoptera species pose significant challenges, leading to economic losses and reduced product quality. By understanding their life cycles and implementing integrated management strategies, farmers can mitigate the impact of these pests and maintain sustainable agricultural practices.

Among Lepidoptera, *Spodoptera littoralis* Boisduval (Lepidoptera: Noctuidae), commonly known as cotton leafworm, is considered a key-pest in the horticulture sector (Noma T. et al., 2010). This pest is widely spread in Africa, Mediterranean Europe and Middle Eastern countries (CABI, 2017; Sidibe B. et al., 1977) and it's included in the EPPO A2 list (EPPO, 2015). In Europe, it is widespread in Spain, Italy, France and Greece. In Italy, it is mainly found in southern regions (Noma T. et al., 2010; Sannino L., 2003).

Spodoptera littoralis is a polyphagous pest of various ornamental plants and horticultural crops able to feed on more than 112 plants belonging to 44 host plant families, including cotton, soybeans,

alfalfa, sweet potato, pepper, eggplant, tomato, maize, lettuce, strawberry, but as well as wheat and hibiscus (Moussa et al., 1960; Hatem et al., 2009; EPPO, 2008; Baskar et al., 2012).

The optimum temperature for this species is 25°C, but it can adapt well to temperature fluctuations and this influences both its reproductive potential and life cycle duration (CABI 2017; Sidibe B. et al., 1977), although temperatures that are too high (>40°C) or too low (<13°C) can cause their mortality (Lopez-Vaamonde C., 2008). Damage is caused by the larvae, which are highly voracious, preferring to feed on young leaves, young shoots, stalks, bolls, buds and fruits (Ismail S., 2019). The damage severity depends on the type of host plant (Kamel SA., 1965); e.g. in Egypt, *S. littoralis* is considered a destructive pest of cotton (Hatem et al., 2009). Due to their polyphagy and the larvae's pronounced voracity, depending on the type of infestation, damage to cultivated plants can be high and consequently also losses of marketable product (Carter D., 1984; Ahmed WH. et al., 2021). Considering the extensive damage and high production losses caused by the larvae attack, pest management is fundamental.

Spodoptera littoralis control is carried out by a series strategies including biological control and IPM and the last seems to be the best one. The effectiveness of these control methods depends on the type of insect, the environment in which it is found and the level of infestation (Vincent C. et al., 2003; Desneux N. et al., 2022). These strategies include the use of pheromone traps to monitor the population, as described by Rizk et al. (1990), but also the use of pheromones for mass trapping, which act as attract and kill (McVeigh LJ. et al., 1987). For example, the installation of 12 sex pheromone traps per ha can be used to monitor the population of *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) and *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae) (Hamed and Nadeem, 2010). The use of trap plants also contributes to moth management. The presence of castor on the borders of vegetable fields reduces the attack of *S. litura* (Sorensen KA. et al., 2016), species

very similar to *S. littoralis*. The genetic selection of varieties tolerant to different pests, or the use of genetically modified plants (GMOs), such as the case of Bt corn plants, i.e. plants containing the *Bacillus thuringiensis* Berliner (Bacillales: Bacillaceae) gene, make plants resistant to attack by the corn borer *Ostrinia nubilalis* Hübner (Lepidoptera: Crambidae) and the corn earworm *Helicoverpa zea* Boddie (Lepidoptera: Noctuidae) (Glaser JA. et al., 2003; Tabashnik BE. et al., 2002).

The agricultural paradigm has historically been characterized by the prevalent application of synthetic insecticides, owing to their perceived efficacy in rapidly mitigating phytophagous insect populations and their extensive market availability across diverse environmental contexts; however, this approach is not without its inherent limitations (Chowański S. et al., 2014) Excessive use of insecticides has also led to the increase of resistance to insecticides in *S. littoralis*, which is resistant to more than 31 active ingredients, including organophosphates, pyrethroids and indoxacarb (Fouad EA. et al., 2022; Arthropod Pesticide Resistance Database, 2021), carbamates and insect growth regulators (IGRs) (Abo-Elghar GC. et al., 1992), but also to newer insecticides such as chlorantraniliprole and bioinsecticides such as *Bacillus thuringiensis* and Spinosad (Hafez SSM. et al., 2018; Fouad EA. et al., 2022; Ahmed MA. et al., 2016).

According to the Arthropod Pesticide Resistance Database 2021, *S. littoralis* is among the top 30 most resistant species in the world (Moustafa MA. et al., 2023).

From this point of view, biological control methods play an important role, which involves the use of insecticides of natural origin, such extracts of fungal or bacterial matrix or viruses. Classical biological control involves the introduction, conservation or massive release of natural enemies (predators, parasitoids and pathogens) into the environment, with the aims to reduce the economic threshold of different pests. Even in the control of the cotton moth *S. littoralis*, there is an interest in implementing biological control. Commercial formulations of *B. thuringiensis* var. *entomocidus* has shown excellent results in terms of mortality of the moth larvae, as it produces lethal toxins that act

in the intestine and lead to the death of the insect (Sneh B. et al., 1981; Navon A., 2000). Among the natural enemies, the generalist predators *Chrysoperla carnea* Stephens (Neuroptera: Chrysopidae) and *Orius levigatus* Fieber (Rhynchota: Anthocoridae), which are known for their predacious activity, can reduce the moth infestation level. Furthermore, the Braconidae family includes parasitoids like *Cotesia marginiventris* Cresson (Hymenoptera: Braconidae), a larval parasitoid that oviposits within *S. littoralis* larvae, ultimately causing host mortality (Hegazi E. et al., 2024; Hill DS., 1987). Similarly, *Chelonus inanitus* Panzer, another braconid wasp, parasitizes lepidopteran eggs, arresting their embryonic development (Kaeslin M. et al., 2005). Another important parasitoid, belonging to the family Trichogrammatidae, is *Trichogramma bactrae* Nagaraja which parasitizes the eggs of *S. littoralis*, reducing the development of the larvae (Hend and Mohamed, 2021). Other promising biocontrol agents are entomopathogenic nematodes (EPNs), such as *Steinernema riobrave* Cabanillas, Poinar & Raulston (Rhabditida: Steinernematidae) and *Heterorhabditis bacteriophora* Poinar (Rhabditida: Heterorhabditidae), which are able to penetrate the body of the larvae, releasing symbiotic bacteria that cause the pest's death (Shaurub ESH. et al., 2016). An important role, in terms of biocontrol, is played by entomopathogenic fungi such as *Bauveria bassiana* Vuillemin (Hypocreales: Cordycipitaceae) and *Metarhizium anisopliae* Mechnikov (Hypocreales: Clavicipitaceae), which penetrate the larvae of *S. littoralis*, causing fungal infections and leading the host to death (El Hussein MMM., 2019).

The use of entomopathogenic viruses, particularly baculoviruses (Baculoviridae), represents another biocontrol strategy. These insect-specific viruses, encompassing over 600 host species (Atia M. et al., 2016), induce nuclear polyhedrosis virus (NPV), resulting in decreased trophic and motor activity in larvae following plant tissue ingestion.

I.II.I.II *Phthorimaea absoluta*

A significant pest of horticultural crops is *P. absoluta*. This invasive microlepidopteran moth, known as the South American tomato pinworm or tomato leaf miner, is widely distributed. It's considered a tomato key-pest and it's a major destructive pest on tomato crops (Roditakis E. et al., 2017; Guedes RNC. et al., 2019), since 1950s (Desneux N. et al., 2010). It's native to South America, while in Europe, its presence was reported in Spain in 2006 and then spread rapidly to the other tomato-producing countries in the Mediterranean area (Biondi A. et al., 2018; Desneux N. et al., 2010; 2011; Tropea Garzia G., 2012). In Italy, *P. absoluta* was first reported 2008, in the southern regions as described by Tropea (2009). This pest has been reported throughout most of Europe and North Africa, and in several Middle Eastern countries (Desneux N. et al., 2011). Before invading Spain, *T. absoluta* was included on the list of quarantine pests of the European Plant Protection Organization but was not listed in the Plant Health Directive (2000/29/EC) of the European Commission and this probably contributed to the introduction of infested plant material into Europe due to the lack of phytosanitary inspection (Biondi A. et al., 2018).

The optimum temperature for this species is 30°C, but it can adapt well to temperature fluctuations between 34.6° and 14°C. This wide temperature range affects both its reproductive potential and the length of its life-cycle, which can last between 26 and 75 days and, thanks to its high adaptability, can complete up to 10 generations in one year (Martins JC. et al., 2016).

Phthorimaea absoluta is not characterized by high polyphagy and it's able to infest, in addition to tomatoes, only other Solanaceae plants such as eggplants, sweet pepper, potato and tobacco, and wild plants such as *Solanum nigrum* Linnaeus (Solanales: Solanaceae) (Zappalà et al., 2012; Desneux N. et al., 2010; Abbas K. et al., 2016). On non-tomato plants, *P. absoluta* causes no notable damage.

Damage is caused by the larvae, which are voracious towards tomato leaves, stems and fruits. They feed on the leaf mesophyll, causing a thin leaf mine (Desneux N. et al., 2010; Martins JC. et al., 2017). The combined effect of *P. absoluta* feeding and subsequent secondary pathogen introduction on tomato plants can lead to severe damage, resulting in product losses between 80% and 100%. (Desneux N. et al., 2011; Cuthbertson AGS. et al., 2013).

Considering the extensive damage and product losses *P. absoluta* inflicts on tomato crops, and the high economic and nutritional value of this vegetable, effective pest management is of paramount importance. As a result, a large volume of scientific literature focuses on developing sustainable control methods. Ex pheromone traps are used extensively for monitoring *P. absoluta* and are intended to improve the sensitivity of existing traps (delta or bucket traps) to early infestations, facilitating the correct implementation of different control methods (Desneux N. et al., 2022; Benvenga SR. et al., 2007).

Commonly used traps are the dark-colored Delta traps (Uchôa-Fernandes MA. et al., 1994; Roda AL. et al., 2015; Abd El-Ghany NM. et al., 2016), which are usually made of paper or plastic in the shape of a triangular prism, inside of which there is an insert of trap board at the base and a pheromone bait suspended under the roof of the trap (Desneux N. et al., 2022). Currently, there are no guidelines on the density of traps to be used, but monitoring is reliable using 1-4 traps/ha (Mansour R. et al., 2019). In case of a mass trapping strategy, the number of traps per ha is higher, approximately 32-36 water traps/ha in open field conditions or 2 delta traps in a greenhouse of 500 m² (Desneux N. et al., 2022; Mansour R. et al., 2019). Usually, mass trapping is not enough to control *P. absoluta* populations and should be used in combination with other measures to achieve an acceptable level of control (Cherif A. et al., 2019; Mansour R. and Biondi A., 2021).

Synthetic insecticides are still widely used today by farmers and represent the fastest way to control the pest, as they have the advantage of drastically reducing the pest's population, also due to the high

number of treatments during the tomato crop cycle, but they are not always the most correct way, due to the emergence of resistance phenomena (Guedes and Picanço, 2012; Guedes and Siqueira, 2012; Campos MR. et al., 2017; Biondi A. et al., 2018; Mansour R. et al., 2018 ; Guedes RNC. et al., 2019; Rwomushana I. et al., 2019).

In Brazil, *T. absoluta* introduction led to a surge in pesticide treatments from 10-12 to 30 (4-6 weekly) per crop cycle (Guedes RNC. et al., 2012). This huge use of conventional treatments (Biondi A. et al., 2018) favored the development of insecticide resistance in *P. absoluta* (Guedes RNC. et al., 2019) and this led to an increase in costs (Potting RP. et al., 2013). Resistance to organophosphates was first reported in South America, such as Chile, Brazil and Argentina (Siqueira HAA. et al., 2000a; Lietti MMM. et al., 2005). The development of pyrethroid resistance has been documented in European populations (Silva GA. et al., 2011; 2015; Haddi K. et al., 2012), while low to medium resistance levels to abamectin, indoxacarb, and diamides have also been reported (Silva GA. et al., 2011; 2016b; Nauen R. et al., 2006). A large number of entomopathogens are lethal to *P. absoluta*, including bacteria, fungi, and nematodes. These microbials are usually not harmful to the environment, they also tend to be safer for humans and other vertebrates and are compatible with other groups of beneficial organisms such as the natural enemies of arthropods (González-Cabrera J. et al., 2011; Mansour R. and Biondi A., 2021).

Some commercial strains of the bacterium *B. thuringiensis* are used as microbial control agents against *P. absoluta*, which are effective if ingested by the larvae (Gonzalez-Cabrera J. et al., 2011) achieving a population reduction of 95%.

Beauveria bassiana, an entomopathogenic fungus, is commonly applied for *P. absoluta* control; however, its effectiveness is constrained, typically resulting in mortalities below 50%, attributed to environmental spore degradation from factors such as UV radiation and rainfall (Klieber JA. and

Reineke A., 2016; Vega FE, 2018). "Entomopathogenic nematodes (EPNs) are also capable of infecting different instars of *P. absoluta* (Batalla-Carrera et al., 2010; Turkoz and Kaskavalci, 2016; Van Damme et al., 2016; Mutegi et al., 2017; Kamali et al., 2018). In particular, entomopathogenic nematodes such as *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* Weiser (Rhabditida: Steinernematidae) are effective for the control of the moth.

Over 160 species of natural enemies of *P. absoluta*, most of them polyphagous, are known in South America and Africa (Desneux N. et al., 2010; Zappalà L. et al., 2013). In Europe, the biological control of *P. absoluta* is carried out through generalist predators, such as *Nesidiocoris tenuis* Reuter (Hemiptera: Miridae) and *Macrolophus pygmaeus* Rambur (Hemiptera: Miridae), which prey on both the eggs and the young larvae of the tomato moth (Jaworski CC. et al., 2015; Molla O. et al., 2011). These two commercially available mirid predatory insects, appear to be effective for biological control of *P. absoluta* in Europe (Pérez-Hedo M. et al., 2021a). Although these two mirids should be released during transplanting and pre-planting activities (Urbaneja A. et al., 2012). Furthermore, numerous parasitoids have been identified on *P. absoluta* in South America, including *Trichogramma* species, which parasitize moth eggs, although this natural parasitism appears to be low. Significantly higher parasitism rates, reaching nearly 90%, have been observed when mass-reared parasitoids are released into tomato greenhouses in Brazil, Spain, and Egypt (El-Arnaouty SA. et al., 2014; Parra JR et al., 2004; Zappalà L. et al., 2013). In Europe, the focus of larval parasitoid studies has been on the Braconidae and Eulophidae families, notably *Necremnus tutae* Nees (Hymenoptera: Eulophidae) (Ferracini C. et al., 2012).

I.II.I.III *Bemisia tabaci*

Among the key pests of different vegetables, whiteflies (Aleyrodidae) due to their rapid reproduction and adaptability making them a constant challenge in agricultural systems. These pests are small, sap-

sucking insects commonly found in greenhouse and field environments. They are particularly notorious for their impact on a wide range of horticultural crops, including tomatoes, peppers, cucumbers, and ornamental plants. Among whiteflies, *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) is one of the most widespread and polyphagous insects in the world of cultivated vegetable plants (Fan W. et al., 2018; Oliveira MRV et al., 2001; De Barro PJ. et al., 2011). It's commonly known as silverleaf whitefly or sweet potato whitefly and it's assumed to be native to the Middle East/Asia Minor. The global distribution of *Bemisia tabaci* is primarily attributed to the widespread commercial trade of infested ornamental plants (Abdullahi I. et al., 2003; Broadbent AB. et al., 1989; Cheek S. et al., 1994).

Bemisia tabaci is characterized by the presence of numerous biotypes, with the most prevalent being A, B, B2, D, E, G, H, J, K, L, M, and Q. In Campania, Italy, biotype T was identified for the first time in 2016 by Parrella et al. (2016). These biotypes are categorized based on specific biological traits, including their ability to transmit begomovirus (Bedford ID. et al., 1994).

The concept of biotype emerged following the invasion of the United States by *B. tabaci*, which exhibited behaviors distinct from the indigenous populations, particularly regarding esterase profiles and host plant preferences. Consequently, these populations were classified into two biotypes: A, representing the indigenous population, and B, denoting the introduced specimens (Bedford ID. et al., 1993; Bird J., 1957; Brown JK. et al., 1992). Currently, biotypes B and Q are the most widely distributed globally (Horowitz AR. et al., 2020; De Barro PJ et al., 2011).

This pest can develop on more than 500 host plants, including ornamental and cultivated plants, (De Barro PJ. et al., 2011; Taggar GK. et al., 2016; Soares M. et al., 2020). In detail, plants belonging to Compositae, Cruciferae, Cucurbitaceae, Solanaceae and Leguminosae families were the preferred host species for *B. tabaci* (Shao-Jian L. et al., 2011).

This whitefly can cause considerable damage to plants through phloem feeding, but the most important issue is the transmission of plant pathogens (e.g. viruses) during the feeding phase (Navas-Castillo J. et al., 2011; Gilbertson RL. et al., 2015).

Bemisia tabaci is a vector for more than 100 plant viruses (Hogenhout SA. et al., 2008) and, in many cases, the transmitted viruses turn out to be plant growth limiting factors and can cause total crop loss (Horowitz AR. et al., 2020). For example, cotton leaf curl virus (CLCV), transmitted by *B. tabaci*, is one of the most important diseases of cotton in Asia and Africa, and tomato yellow leaf curl virus (TYLCV) is one of the most damaging viruses affecting tomato crops worldwide (Horowitz AR. et al., 2020).

The honeydew excretion of *Bemisia tabaci* triggers a cascade of negative effects: promoting opportunistic fungal growth, diminishing plant photosynthesis, and ultimately culminating in yield loss (De Barro PJ. et al., 2011; Gilbertson RL. et al., 2015; Boykin LM. et al., 2013). Although *Bemisia tabaci* is globally distributed and highly adaptable, its oviposition rate is maximized at 28°C (Sani I. et al., 2020). The polyphagous nature of *Bemisia tabaci*, coupled with its substantial primary and secondary damage to horticultural crops and its notable resistance to insecticides, underscores the critical need for robust pest management strategies. The IPM program for *B. tabaci* includes biological control, physical and mechanical methods, and the use of selective chemical pesticides if necessary (Horowitz AR. et al., 2011). A widespread technique among farmers to counter the attack of the whiteflies is based on mass capture with adhesive traps, with yellow sticky surfaces (Lu Y. et al., 2012); however, these types of traps are also important tools for monitoring whitefly populations (Gerling D. and Horowitz AR., 1984).

Driven by the extensive damage and yield losses inflicted by *Bemisia tabaci*, farmers have resorted to numerous chemical insecticide treatments, inadvertently promoting the development of resistance. It is known that *B. tabaci* is resistant to a large class of active ingredients, such as organophosphates,



carbamates (Horowitz AR. et al., 2007; Naveen NC. et al., 2017), pyrethroids (Naveen NC. et al., 2017; Roditakis E. et al., 2009) such as bifenthrin and cypermethrin, but also growth regulators (IGRs) such as pyriproxyfen and buprofezin (Horowitz AR. et al., 2005; Roy S. et al., 2019) and neonicotinoids. In particular, biotype Q appears to be more resistant than biotype B (Horowitz AR. et al., 2005; Yao FL. et al., 2017). According to Arthropod Pesticide Resistance Database (Michigan State University), there have been approximately 650 reported cases of insecticide resistance on whitefly, and such resistance has been detected to over 60 active ingredients (Mota-Sanchez D. et al., 2019).

Control of *B. tabaci* can be effectively carried out by integrating multiple biological control agents such as parasitoids, predators and Entomopathogenic Fungi (EPF) (Colmenarez Y. et al., 2018; Rodríguez E. et al., 2019; Singh D. et al., 2017). In fact, in literature, there are at least 115 species of whitefly parasitoids belonging to five main families such as: Aphelinidae, Azotidae, Encyrtidae, Signiphoridae (Chalcidoidea) and Platygasteridae (Platygastridae) (Lahey Z. et al., 2016). Two genera, such as *Encarsia* and *Eretmocerus* belonging to Hymenoptera order, appear to be the most spread whitefly parasitoids throughout the world (Shah MMR. et al., 2015). Most predators of *B. tabaci* belong to the family Coccinellidae, Miridae Anthocoridae, Chrysopidae and Phytoseiidae mites (Gerling D. et al., 2001), in particular the mite *Amblyseius swirskii* Athias-Henriot (Mesostigmata: Phytoseiidae), can be considered among the most important (Horowitz AR. et al., 2020). A widespread practice for the biological control of greenhouse tomato whiteflies in Europe is the inoculative release of *N. tenuis* and *M. pygmaeus* predators at a density of 1-2 individuals/m², 3-4 weeks after transplanting (Gabarra R. et al., 2006; Calvo J. et al., 2009). Entomopathogenic fungi represents a group of biocontrol agents effective against whiteflies (Sani I. et al., 2020), as they manage to infect insects directly through the cuticle (Mascarin GM. et al., 2013).



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Most of the EPFs involved in the control of *B. tabaci* were species of the following genera: *Aschersonia*, *Beauveria*, *Isaria* (Paecilomyces), *Lecanicillium* (formerly *Verticillium*) and *Metarhizium* (Perring TM. et al., 2018). Currently, *B. bassiana* was developed commercially as a microbial insecticide to control *B. tabaci* and is one of the most widely used (Perring TM. et al., 2018) and it is effective against eggs and the second nymphal instar of *B. tabaci* on four host plants: *Gossypium hirsutum* L. (Malvales: Malvaceae), *Lycopersicon esculentum* L. (Solanales: Solanaceae), *Solanum melongena* L. (Solanales: Solanaceae), and *Capsicum annum* L. (Solanales: Solanaceae) (Sani I. et al., 2020).

I.III Evolution of the use of chemical insecticides

While pesticides remain a critical tool for farmers in controlling agricultural pests and supporting global living standards (Popp J. et al., 2013), their substantial environmental and social costs necessitate continued attention (National Research Council, 2000). Over the past 30 years, the global use of chemical insecticides has undergone significant transformations, marked by changes in substance types, regulatory frameworks, and sustainable management approaches. These shifts have been driven by growing awareness of environmental impacts, risks to human and animal health, and the development of insect resistance.

A primary change has been the reduction of highly toxic insecticides, such as organochlorines (e.g., DDT). Although bans began in many countries during the 1970s, the elimination process continued into the 1990s and beyond in other regions. Many developing countries have reduced or eliminated these insecticides through international regulations like the Stockholm Convention on Persistent Organic Pollutants (POPs), which took effect in 2004 (Matthews GA., 2016; Simon-Delso et al.,

2015). Alongside organochlorines, highly toxic organophosphates and carbamates have been progressively replaced by less hazardous substances due to their significant risks to human and animal health.

Concurrently, the adoption of less persistent and more targeted insecticides, including pyrethroids and neonicotinoids, increased significantly from the 1990s onward. Pyrethroids, with lower mammalian toxicity and reduced persistence compared to organophosphates, experienced substantial growth in agricultural use. However, neonicotinoids faced increasing controversy in the 2010s due to their detrimental effects on pollinators, particularly bees. This led to significant regulatory restrictions, notably in Europe, where organophosphates (e.g., chlorpyrifos and chlorpyrifos methyl) and neonicotinoids (e.g., imidacloprid and thiamethoxam) were banned in 2018 and 2020, respectively (EC Regulation 1107/2009, Official Journal of the European Union, 2020; Simon-Delso et al., 2015).

European Directive 91/414/EEC initially established insecticide registration and use procedures, subsequently superseded by EC Regulation 1107/2009, which took effect in June 2011 and mandates compliance across all EU member states. This regulation aims to minimize environmental pollution by evaluating manufacturer-provided data and eliminating the most hazardous substances (Matthews GA., 2016).

Over the past 30 years, a trend has emerged towards developing more selective and biodegradable insecticides, with reduced impact on non-target organisms and the environment. Biotechnology has facilitated the creation of new insecticide classes, such as insect growth regulators (IGRs), which operate by disrupting insect growth or reproduction, thereby reducing risks to non-target animals (Matthews GA., 2016; Popp J. et al., 2013).

This trend aligns with the adoption of integrated pest management (IPM) strategies. Promoted and implemented globally, IPM integrates insecticide use with biological, cultural, and mechanical



control techniques. This approach aims to minimize chemical use, enhance agricultural sustainability, and prevent resistance development. IPM is now widely supported by international organizations like the FAO and adopted as official agricultural policy in many countries.

Over the past 20 years, IPM programs have reduced agricultural insecticide use in fruit and vegetable crops, consequently lowering pest control costs. These reductions are attributed to the introduction or increase of natural enemies, the selection of resistant varieties, optimized cultural practices, and the judicious use of alternative insecticides (Popp J. et al., 2013). Directive 2009/128/EC of the European Commission on the sustainable use of pesticides promotes sustainable pesticide use by minimizing risks to human health and the environment, and by encouraging the adoption of IPM and alternative techniques (Popp J. et al., 2013).

I.IV. Botanical insecticides

Taking into consideration the problems linked to the use of insecticides of synthetic origin on the environment and the ecosystem, on the development of resistance by plant phytophagous insects, the negative effects on non-target organisms, the health of farmers and consumers and of socioeconomic costs, we can ask ourselves the question: is there any alternative to the use of pesticides? (Chowański S. et al., 2014).

Botanical extracts are increasingly used as natural repellents and insecticides. Botanical extracts offer several advantages: they are generally biodegradable and do not accumulate harmful residues in soil or water, minimizing environmental impact. Furthermore, they exhibit lower toxicity to humans and animals compared to synthetic pesticides. Additionally, the slower development of insect resistance to botanical extracts enhances their long-term sustainability (Regnault-Roger C. et al., 2012).

Botanical insecticides, are getting more and more attention from researchers, environmentalists and farmers, as they are considered viable alternatives to the use of chemical insecticides (Pavela R. et al., 2016; Campolo O. et al., 2020; Ben Abdallah S. et al., 2023), which contribute to avoid multiple negative factors on non-target insects, water and the environment, biodiversity, as well as favoring the emergence of resistance phenomena by harmful insects (Naqqash MN. et al., 2016).

Over time, plants have evolved diverse physical and chemical defenses against insects, likely as a response to insect herbivory. These defensive compounds, including phenols, polyphenols, terpenoids, and alkaloids, can be isolated through various extraction techniques. These techniques range from simple maceration of plant material in water to extractions using organic solvents or various distillation methods. Some of these extracts serve as active ingredients in botanical insecticides (Pavela R., 2015a; Regnault-Roger C. et al., 2012) From a commercial point of view, probably the most widely used botanical insecticide (BIs) is the so-called Neem oil, which is extracted from the *Azadirachta indica* Jussieu (Sapindales: Meliaceae) seeds, composed mainly of Azadirachtin (Pavela, 2016), which is known for its effectiveness against the juvenile stages of insects (Dwivedi N., 2008). Among the most marketed botanical insecticides are those based on *Allium sativum* L. (Asparagales: Amaryllidaceae), consisting mainly of sulphur compounds and those based on *Citrus sinensis* Osbeck (Sapindales: Rutaceae), consisting mainly of limonene (Pavela, 2016). Other commonly used products are based on extracts from *Chrysanthemum cinerariifolium* Treviranus (Asterales: Asteraceae) flowers, composed mainly of pyrethrins, which are neurotoxic in that they block sodium and potassium channels, interrupting normal nerve transmission, and thus have a knock-down action against insects (Pavela, 2016). BIs based on pyrethroids are commonly used against small phytophagous insects such as aphids, whiteflies, red spider mites, thrips, flies and mosquitoes.

I.IV.I. Essential oils (EOs) as insecticides

Another very important and commonly used group within botanical insecticides is represented by essential oils (EOs), which are considered important secondary metabolites of plants effective against phytophagous insects and are also available throughout the world and relatively cost-effectiveness (Pavela R., 2016; Campolo O. et al., 2018).

Essential oils are synthesized by plants both internally, through the secretory glands located inside the plants, and externally, through the external glands. EOs are obtained from different plant organs such as flowers, herbs, shoots, leaves, fruits, twigs, bark, seeds, wood, rhizomes and roots. The plant species that produce essential oils are widely distributed throughout the world and belong to a limited number of families such as: Asteraceae, Cupressaceae, Lamiaceae, Lauraceae, Rutaceae, Myrtaceae, Piperaceae and Poaceae (Pavela R., 2015; Zuzarte M. et al., 2015; Svoboda K. et al., 2003; Asbahani AE. et al., 2015; Fahn A., 2000; Bruneton J., 1999; Boate UR. et al., 2020). Generally, EOs are made up of a mixture of 20-70 organic compounds, such as monoterpenes, phenols, and sesquiterpenes, some of which represent more than 80% of the constituents (Pavela R., 2016). For example, in sweet orange EO, the main compound is limonene, which makes up 88-97% of the whole oil (Campolo O. et al., 2014; Campolo O. et al., 2016). Instead, in the case of garlic essential oil, 44 compounds were found, of which 96.3% are sulfur compounds, such as diallyl disulfide (37.26%) (Modafferi A. et al., 2024).

From a physical point of view, EOs are characterized by a strong odor and they are hydrophobic and generally lipophilic and are soluble in organic solvents (Khater H., 2012; Campolo O. et al., 2018). The multifaceted activity of essential oils encompasses direct insecticidal toxicity, antifeedant effects, repellency, and the attraction of specific non-target insects (Khater H., 2012; Campolo O. et al., 2018; Pavela R., 2015). They are widely used as fumigants against stored insects (Campolo O. et al., 2018).

In addition, the use of essential oils can also cause sub-lethal effects on insects, such as changes in reproductive performance and development with specific reference to growth rate, food consumption and food utilization (Campolo O. et al., 2018).

The application of essential oils as botanical insecticides offers numerous advantages, including low or negligible toxicity to non-target organisms. In fact, fields treated with plant extracts often exhibit a higher abundance of beneficial organisms compared to those treated with synthetic insecticides. (George DR. et al., 2010; Issakul K. et al., 2011; Pavela R. et al., 2013a, 2014a; Amoabeng BW. et al., 2013; Mikenda P. et al., 2015). Botanical extracts offer the advantage of rapid residue degradation (Fernandez-Perez M. et al., 2015; Flores-Céspedes F. et al., 2015), resulting in safety for human health, environmental preservation, and ecosystem integrity. This safety profile is attributed to the low or non-existent residues deposited on leaves and fruits (Boate UR. et al., 2020; Talukder F. et al., 2004; Isman MB. et al., 2020). These positive aspects confirm the importance of using essential oils as alternatives to the use of chemical insecticides (Pavela R., 2016). In fact, the number of scientific articles focused on the insecticidal efficacy of these botanical insecticides against harmful insects and nematodes belonging to different orders and species is constantly growing (Giuliano G. et al, 2024; Modafferi A. et al., 2024a; Campolo O. et al., 2018; Campolo O. et al., 2017; Ben Abdallah S. et al., 2023; Erdemir T. and Erler F., 2017, 2018; Hamada HM et al., 2018; Catani L. et al., 2023; Jardim IN. et al., 2020).

I.IV.II Nanoemulsion

The use of essential oils, such as bio-insecticide, in agriculture is increasingly popular due to their natural properties such as insecticides, fungicides and repellents. However, there are also some negative effects that must be considered to avoid undesirable impacts on the environment, crops and



operators. Essential oils, due to their volatility and thermolability, degrade easily and this factor is influenced by various chemical and environmental factors, such as temperature, light and oxygen availability. In addition, the presence of impurities in the EO, as well as their nature and structure, can be decisive for the stability of the EO (Turek C. et al., 2013; Odak I. et al., 2018).

Essential oils, as such, are losing their insecticidal efficacy when applied in the field, probably due to critical issues such as volatility, thermolability, poor solubility in water and their rapid degradation (Raveau R. et al., 2020; Campolo O. et al., 2020; Krzyżowski M. et al., 2020).

Furthermore, EOs can be phytotoxic to plants if used in high concentrations, indeed many of them are used for weed control (Campolo O. et al., 2020; Karalija E. et al., 2020). Despite their advantages, the application of some essential oils can result in phytotoxicity, characterized by symptoms such as leaf burn, necrosis, and diminished plant growth, ultimately impacting yield (Ibáñez MD. et al., 2020). The occurrence and severity of phytotoxicity are modulated by crop susceptibility, prevailing environmental conditions (including temperature and humidity), and the specific application technique employed. To overcome these negative characteristics, EOs can be encapsulated within nanostructures (e.g. nanoparticles and nanoemulsions), which can ensure the preservation of insecticidal properties, improve the stability and the water solubility of the formulation (Campolo O. et al., 2017; Giunti G. et al., 2019; 2023; Campolo O. et al., 2020; Raveau R. et al., 2020). Nanoemulsions usually involve mixing oil in water (O/W) or water in oil (W/O), with the addition of non-ionic surfactants, which act as stabilizers (Campolo O. et al., 2020; Raveau R. et al., 2020; Pascual-Villalobos MJ. et al., 2019). These nanoemulsions can be obtained through different techniques, based on the use of high energy, such as high-pressure or mechanical homogenization and ultrasound, or using low-energy techniques, such as isothermal and thermal processes (Aswathanarayan JB. et al., 2019; Komaiko JS. et al., 2016). The choice of surfactant and the method



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of obtaining the nanoemulsions, represents a fundamental factor (Raveau R. et al., 2020; Pavoni L. et al., 2019).

Key factors in nanoemulsion development include particle size (nm), polydispersity index (PDI), and surface charge (ζ). Nanoemulsions typically exhibit particle sizes between 50 and 200 nm (Tadros T. et al., 2004). This small particle size confers long-term stability by preventing flocculation (Campolo O. et al., 2020). Additionally, nanoemulsion formulations require less surfactant, possess a uniform surface, and demonstrate enhanced wetting, distribution, and penetration properties (Bouchemal K. et al., 2004).

II. Aims of thesis

The challenges associated with synthetic insecticide use in agriculture for pest control, coupled with the withdrawal of certain products from the market, have driven research into alternative solutions. Among these, essential oils have gained significant traction (Campolo O. et al., 2020; Raveau R. et al., 2020; Giuliano G. et al., 2024).

This thesis aimed to evaluate the insecticidal efficacy of garlic essential oil-based nanoformulations, produced using high-energy methods at varying pressures (2000, 20000, and 30000 PSI), against three major vegetable pests: the cotton bollworm *S. littoralis*, the tomato leaf miner *P. absoluta*, and the silverleaf whitefly *B. tabaci*. High-energy methods are known to generate more stable nanoformulations, facilitate the use of higher active ingredient (ai) concentrations, and require less surfactant compared to low-energy methods (Modafferi A. et al., 2024).

Specifically, the study sought to assess the potential insecticidal efficacy of these formulations against different life stages of the three target insects.

Additionally, the potential phytotoxic effects of garlic nanoemulsions were evaluated on pepper plants under semi-field conditions and on tomato, eggplant, cucumber, and pepper plants maintained under controlled climatic conditions.

Furthermore, the impact of garlic formulations produced at 2000 and 20000 PSI was assessed on the generalist predator *N. tenuis* using two distinct application methods, to evaluate the formulations' potential effects on a non-target insect.



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III. Insecticidal Activity of *Allium sativum* Essential Oil-Based Nanoemulsion against *Spodoptera littoralis*

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Simple Summary

Spodoptera littoralis, the Egyptian or African cotton leafworm, is a major agricultural pest in Africa, Mediterranean Europe, and the Middle East, affecting crops like cotton, soybeans, and tomatoes. This pest damages various plant parts, leading to significant product losses. Control strategies primarily use synthetic insecticides, which pose problems such as resistance, environmental harm, and negative effects on non-target organisms. This study explores a garlic essential oil-based nanoemulsion as an alternative control tool. Garlic-EO nanoemulsion effectively killed larvae and reduced feeding

activity in laboratory trials. These findings highlight the potential of botanical insecticides for sustainable pest management.

Abstract

Spodoptera littoralis, commonly known as the Egyptian or African cotton leafworm, is a significant agricultural threat. It is widely distributed in Africa, Mediterranean Europe, and Middle Eastern countries. This polyphagous pest infests numerous crop plants across 44 families, including cotton, soybeans, alfalfa, sweet potato, pepper, eggplant, tomato, maize, lettuce, strawberry, wheat, and hibiscus. The damage caused by *S. littoralis* on different plant organs, such as young leaves, shoots, stalks, bolls, buds, and fruits, often determines substantial product losses. Current control strategies predominantly rely on synthetic insecticides, which, despite their efficacy, have notable drawbacks, including insecticide resistance, environmental contamination, consumer concerns, and adverse effects on non-target organisms and beneficial insects. In response to these challenges, in this study, we developed and evaluated a garlic EO-based nanoemulsion with a high EO concentration (15%) and low surfactant content to mitigate the possible negative impact on plants and to enhance efficacy against *S. littoralis* larvae. Laboratory bioassays demonstrated promising larvicidal activity and reduced larval feeding, although some phytotoxicity symptoms were observed. This study underscores the potential of botanical insecticides as sustainable alternatives to synthetic chemicals, emphasizing the importance of balancing efficacy with environmental and ecological considerations in pest management strategies.

Keywords: antifeedant; biopesticides; cotton leafworm; garlic; phytotoxicity

1. Introduction

Spodoptera littoralis Boisduval (Lepidoptera: Noctuidae), known as the Egyptian or African cotton leafworm, is widely distributed in Africa, Mediterranean regions of Europe and Middle Eastern countries [1]. This moth is a polyphagous pest of various ornamental plants and horticultural crops, infesting dozens of cultivated plants belonging to 44 families, including cotton, soybeans, alfalfa, sweet potato, pepper, eggplant, tomato, maize, lettuce, and strawberry, as well as wheat and hibiscus [2]. Damage is caused by larvae trophic activity, which feed on young leaves, shoots, stalks, bolls, buds, and fruits [3]. Its infestation can also produce the subsequent development of pathogens on damaged plant tissues and can lead to significant product losses [4]. Nowadays, the most common control strategies used against this pest rely on applying synthetic insecticides. Different groups of insecticides, such as organophosphates, pyrethroids, carbamates and insect growth regulators (IGRs), as well as several newer insecticides, including indoxacarb and chlorantraniliprole, are commonly used to control *S. littoralis* [5]. However, these approaches present some drawbacks, as those synthetic molecules can contribute to the emergence of insecticide resistance, environmental pollution, consumer rejection, and adverse effects on non-target organisms and beneficial insects [6,7]. Resistance has already been proved for most of the above-cited synthetic insecticides, mainly due to the development of acetylcholinesterase enzyme (AChE) insensitivity or metabolic detoxification enzymes [8]. The eco-toxicological, environmental, and social implications arising from the extensive and sometimes indiscriminate use of synthetic insecticides in agriculture have encouraged researchers to find more sustainable alternatives to conventional pesticides. Bioinsecticides, including *Bacillus thuringiensis*, spinosad, and spinetoram, are commonly applied in fields to prevent *S. littoralis* damage, although resistance toward those bioactive substances has already been recorded in wild pest populations [8]. Among these alternatives, botanical extracts, particularly essential oils (EOs), have emerged as a promising option for pest control. Their potential lies in their wide availability and

relative affordability, as well as in their intrinsic insecticidal properties, which derive from the defensive role of these molecules in plant physiology [9].

One of the primary advantages of EOs is their acknowledged low toxicity to humans and non-target organisms. Unlike conventional synthetic insecticides, which often pose health and environmental risks, these blends of compounds are generally considered safe when used appropriately. They offer a sustainable and eco-friendly approach to pest management, aligning with integrated pest management (IPM) principles and organic farming practices [10]. Furthermore, EOs can exhibit broad-spectrum activity, targeting multiple pests across different insect orders. Their complex chemical compositions often include various bioactive compounds that act synergistically, reducing the possibility of insects establishing resistance. These characteristics make EOs valuable tools for controlling pest populations and reducing reliance on synthetic chemicals [11]. Moreover, EOs can offer additional benefits beyond insecticidal activity. Some of them possess repellent properties, deterring insects from treated areas, while others may exhibit antimicrobial or antifungal effects, reducing the risk of secondary infections by plant pathogens [12]. However, it is crucial to acknowledge the limitations associated with EO-based insecticides. One key drawback is their relatively short persistence since EO volatile compounds evaporate or degrade rapidly, limiting their residual activity. Frequent reapplication may be necessary, which can be labor-intensive and increase production costs [13]. In this context, the proper development and evaluation of the insecticidal efficacy of plant molecules or extracts could play an important role in developing innovative techniques and tools for controlling this key pest [14].

The main objective of this study was to investigate the insecticidal activity of garlic EO against *S. littoralis* larvae. Garlic, *Allium sativum* L. (Amaryllidaceae), is a globally cultivated crop primarily used in the food and pharmaceutical industries. In the literature, the efficacy of garlic EO has been

investigated against several pests. Indeed, *A. sativum* EO possesses a recognized nematocidal activity [15], and its major constituents (organosulfur compounds) could act as acaricides against eriophyid mites [16,17] and as insecticides against stored product and crop insect pests [18,19]. The large availability, coupled with the promising bioactivity of either garlic aqueous extracts or EOs, made this plant a key candidate for developing innovative eco-friendly insecticides [20,21]. In this context, we have developed a garlic EO-based nanoemulsion characterized by a high amount of active ingredient (i.e., 15% of EO) and a low content of surfactant (Tween 80). The formulation underwent physical characterization using a dynamic light scattering (DLS) apparatus. The study aimed to evaluate the mortality rates induced by the formulated garlic EO, and to estimate the lethal concentrations (LCs) causing 50% and 90% mortality in the tested insect population, its phagodeterrent effects against *S. littoralis* larvae, and the possible phytotoxic impact on target plants.

2. Materials and Methods

2.1. Insects and Plant Rearing

The laboratory strain of *S. littoralis* used in this study was sourced from the agricultural research center Agrigeos s.r.l. (Acireale, Italy). The larvae were reared under laboratory conditions on an artificial diet (41.4 g/L wheat germ, 59.2 g/L brewer's yeast, 165 g/L cornmeal, 5.9 g/L ascorbic acid, 1.53 g/L benzoic acid, 1.8 g/L methyl 4-hydroxybenzoate and 29.6 g/L agar), as suggested by Caccia et al. [22], while the adults were provided with a sugar solution (1:1 w:w). All experiments used bell pepper plants (*Capsicum annuum* L., cv Makko F1). The plants used in the study were grown from untreated seeds and planted in pots filled with organic soil. The plants were regularly watered throughout the growth period and received the required nutrients. Both the insects and the plants used in the experiments were maintained under controlled constant conditions, with a temperature of 24 ± 2 °C, relative humidity (RH) of $50\% \pm 10$, and a photoperiod of 14:10 h (L:D).

2.2. Development and Characterization of Garlic EO-Based Nano-Formulation

To assess the effectiveness of garlic EO against *S. littoralis* larvae while mitigating potential negative effects on plants (i.e., phytotoxicity), an insecticide nano-formulation was developed following the methodology described by Palermo et al. [19]. The developed formulation was characterized by a high amount of EO (i.e., 15%) and relatively low content (i.e., 5%) of surfactant (Tween 80) with an oil:surfactant ratio of 3:1. The preparation process involved mixing 15 g of garlic EO with 5g of Tween 80 using a magnetic stirrer (7000 RPM) for 30 min. Subsequently, distilled water (80 g) was slowly added to the mixture at 1 mL per minute. The resulting coarse emulsion was stirred for three hours (7000 RPM) and then sonicated (100 W for 90 s for 3 cycles) to enhance its stability and homogeneity. To avoid degradation of the garlic EO caused by the heat generated during sonication, the entire process was carried out using an ice bath. The raw garlic EO used in the experiments was purchased from Esperis s.p.a. (Milan, Italy) and belonged to the same batch (OL.ES. 4 20/2) as that used by Modafferi et al. [23], which provided complete analytical procedures and chemical characterization. A Dynamic Light Scattering (DLS) apparatus (Zetasizer Nano, Malvern, UK) was used to analyze the physical characteristics of the developed garlic EO-based nanoemulsion. The mean droplet size, the polydispersity index (PDI), and the droplet surface charge, indicated by the zeta potential (ζ) values, were assessed by diluting the nanoemulsion with double-distilled water (1:400 v:v).

2.3. Laboratory Bioassay on *S. littoralis* Larvae

The experiments were carried out at the laboratories of General and Applied Entomology of the Department of Agriculture of the University of Reggio Calabria (Italy) under controlled environmental conditions in growth chambers (25 ± 2 °C, $60 \pm 10\%$ RH, 14:10 h L:D). Seven different concentrations of garlic EO (0, 0.5, 1, 1.5, 2, 2.5, and 3%) were tested, and water was used as negative

control. Based on preliminary observations, the tested concentrations were chosen to identify the dosage resulting in mortality levels ranging between 10% and 90% of the exposed larvae. This mortality range was selected to accomplish the Probit analysis requirement to adequately calculate the concentration of EO required to kill 50% (LC50) and 90% (LC90) of the exposed larvae. Chlorantraniliprole (Altacor®, DuPont Mississauga, Mississauga, ON, Canada) was used as a positive control. *Spodoptera littoralis* larvae were exposed to the maximum label dose for peppers (12 g/hL Altacor®, corresponding to 0.042 g/L of chlorantraniliprole).

The insecticidal activity of the developed formulation was assessed against the second instar larvae of *S. littoralis* using the leaf dip method. In detail, fresh bell pepper-leaf discs with a diameter of 5.2 cm were dipped into the different dilutions of the nano-formulation for 10 s and left to air dry for 45 min. After drying, each treated leaf disc was carefully placed in a sterile plastic arena (5.5 × 3.5 cm). Afterward, five larvae were released into each arena using a fine paintbrush. Once the larvae were placed inside the arena, the arenas were covered with a mesh to avoid any escape, allow ventilation, and prevent fumigation effect. For each concentration tested, including the controls (water and chlorantraniliprole), six replicates (arenas) were provided. Mortality was recorded daily for three days following the treatment. The larvae were considered dead if they did not display any movement upon stimulation with a fine paintbrush. In addition, the leaf surface eroded by larvae during the experiments was evaluated by ImageJ® v.1.53 software (National Institutes of Health, Bethesda, MD, USA) and expressed as a percentage of eaten leaf disc.

2.4. Nontarget Effects on Plants

A specific trial was set up to evaluate the effects of the EO-based nano-formulation treatments on plants. This experiment was carried out at the agricultural research center Agrigeos s.r.l. (Acireale,

Italy), in an experimental greenhouse. Fifteen days after plant emergence, these were transplanted into pots (two plants/pot) and fertilized using a nitrogen-based fertilizer.

Bioassays were carried out by spraying plants once until runoff, with the most effective concentrations tested against the second instar larvae of *S. littoralis* (i.e., 2, 2.5, and 3%). Similarly to the pest bioassays, water was used as negative control. Each treatment was replicated six times.

The number of healthy and damaged leaves and fruit, the severity of damage, and the plants' height were recorded and counted for each pot; the amount of chlorophyll contained within the leaves, as measured by the SPAD-502Plus (Konica-Minolta, Osaka, Japan) optical sensor, was also registered.

The effect of the developed formulation on plants was estimated using the phytotoxicity index (P_i) proposed by Campolo et al. [14] and calculated as follows:

$$P_i = \sum_{j=0}^n \left(\frac{DL_j}{TL} \times \frac{DC}{n-1} \right)$$

where DL is the number of damaged leaves for each damage severity class j , TL is the total number of leaves sprayed, DC is the damage severity class, and n is the number of damage-severity classes (0 = no phytotoxicity damage; 1 = 25% of damaged leaf surface; 2 = 50% of damaged leaf surface; 3 = 75% of damaged leaf surface; and 4 = 100% of damaged leaf surface). The calculated P_i ranges from 0 (no damage) to 1 (dead leaves). Plant damage was estimated daily for the first 7 days after treatment. After this period, damage was evaluated every 7 days. At the end of the experiments (i.e., 52 days), the plants were cut and weighed with and without fruit. Additionally, the radical apparatus was weighed by first removing the soil from the roots.

2.5. Data Analysis

Statistical analyses were carried out by using IBM SPSS 19 (Supplemental Materials S1). Mortality rates, eroded leaf surfaces from *S. littoralis* bioassays, and data from phytotoxicity trials were

subjected to ANOVA statistical procedure followed by HSD Tukey post hoc. All the data satisfied the ANOVA assumptions in terms of normality and homoscedasticity of variance ($p > 0.05$). Probit analysis was used to estimate LC_{50} s and LC_{90} s. Values were significantly different if their 95% fiducial limits did not overlap. This analysis provided insights into the potency and efficacy of the formulation. The obtained results were interpreted based on p -values ($\alpha = 0.05$).

3. Results

3.1. Characterization of Garlic EO Nanoemulsion

The developed formulation was characterized by an average particle size of 141.0 ± 1.375 nm and a relatively low (0.146 ± 0.009) polydispersity index (PDI), suggesting a relatively narrow size distribution and excellent uniformity. In addition, the nanoemulsion exhibited a surface charge of -27.4 ± 1.91 mV, indicating the presence of a negative charge on the particle surfaces. During the experiments, no evidence of phase separation of the developed nano-insecticide was highlighted, confirming the stability of the nanoemulsion system (Figure 1 and Figure 2).

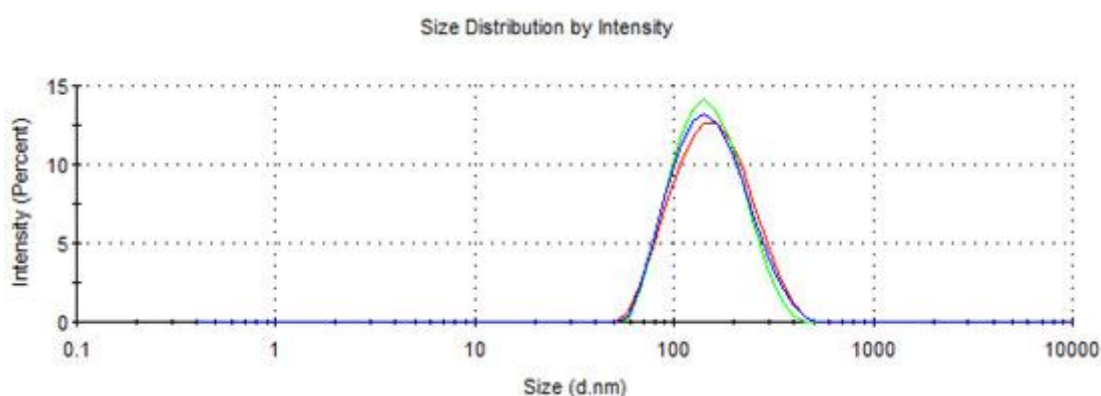


Figure 1. Size distribution of garlic EO-based formulation. Different colors represent different replicates.

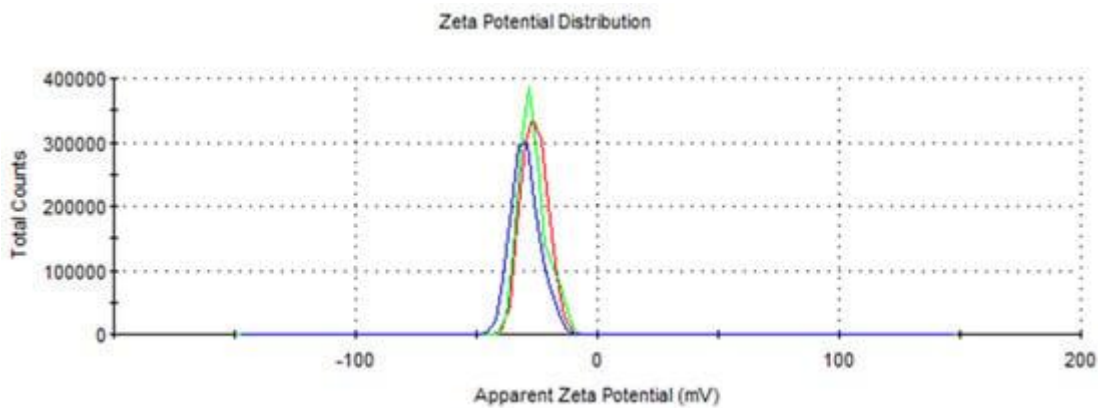


Figure 2. Zeta potential (ζ) of garlic EO-based formulation. Different colors represent different replicates.

3.2. Laboratory Bioassays

The garlic EO-based nano-formulation exhibited high toxicity against second-instar larvae of *S. littoralis*. No mortality was recorded in the untreated control during the entire observation (72 h), whereas 100% mortality was recorded in the positive control.

The mortality induced by the developed formulation was significantly different among the application rates used ($F = 86.70$; $df = 8$; $p < 0.01$), whereas the time from the treatments did not affect the number of dead larvae ($F = 1.623$; $df = 8$; $p = 0.21$). The data related to mortality fitted the probit model (Table 1), and the results of the different LC values confirm that the efficacy of the developed formulation exerts its larvicidal action mainly during the first 24 h after treatment. Indeed, the LC values highlighted no statistical differences among the three different exposure times (i.e., fiducial limits did not overlap). The amounts of EO required to kill 50 and 90% of the exposed larvae after 24 h were 1.72 and 2.79%, respectively.

Table 1. Acute toxicity of garlic EO nanoemulsion against the second instar larvae of *S. littoralis* 24, 48, and 72 h after the exposure. Values are expressed in percentage of EO and are considered significantly different if their 95% fiducial limits (i.e., values shown in parenthesis) do not overlap.

Time	LC ^a ₅₀ (FL) ^b	LC ₉₀ (FL)	χ^2 ^c (df ^d)	<i>p</i> ^e
24 h	1.72 (1.56–1.88) ^a	2.79 (2.48–3.31) ^a	33.58 (46)	0.91
48 h	1.57 (1.43–1.70) ^a	2.39 (2.15–2.76) ^a	41.35 (46)	0.67
72 h	1.57 (1.43–1.70) ^a	2.39 (2.15–2.76) ^a	41.35 (46)	0.67

^a Lethal Concentration; ^b Fiducial limits; ^c Pearson goodness of fit test; ^d Degree of freedom; ^e Probability value.

The antifeedant effect of the garlic EO-based formulation is depicted in Figure 3 and Figure 4. The eroded leaf surface after the treatment followed a dose-dependent pattern ($F = 336.51$; $df = 7$; $p < 0.001$), where larvae were able to eat more than 50% of the untreated leaf area. Larvae treated with the maximum application rate (3% of EO) did not feed on leaves.

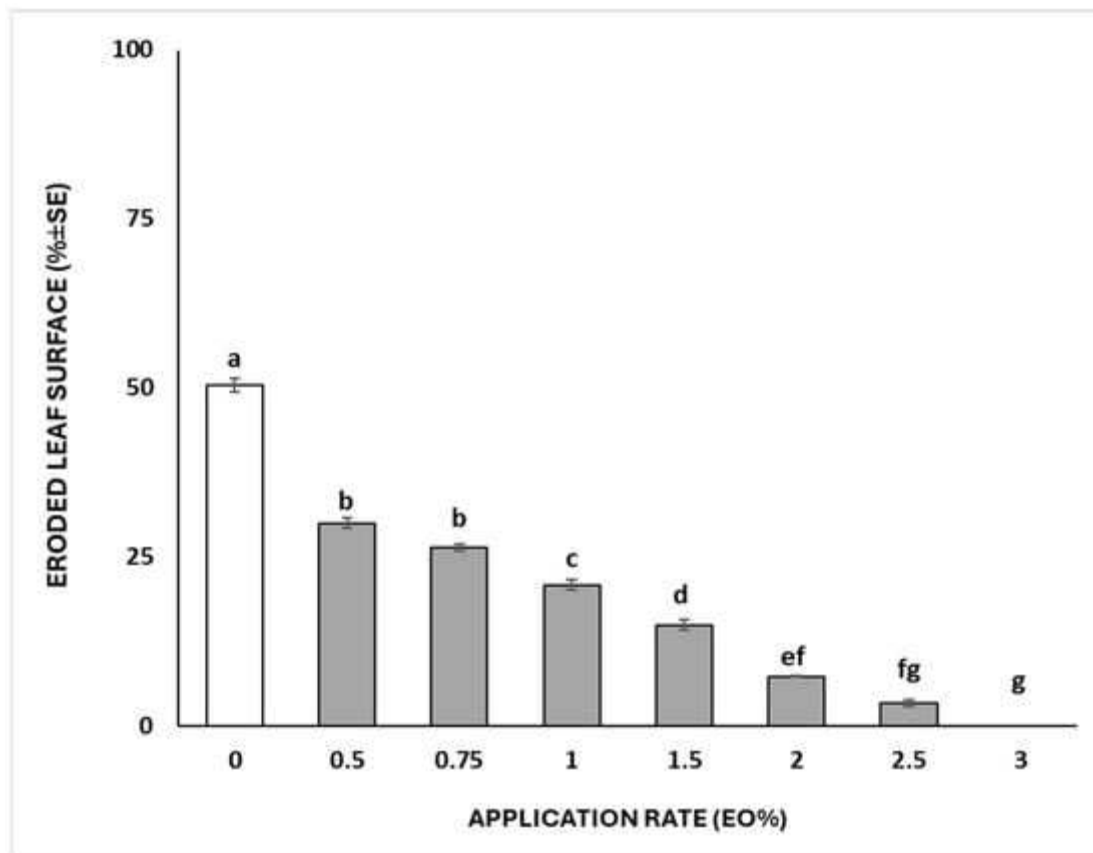


Figure 3. Percentage (mean ± SE) of disc leaf eroded by *S. littoralis* second instar larvae 72 h after the treatments. Different letters indicated significant differences among the groups according to Tukey's HSD post hoc test at $p \leq 0.05$.

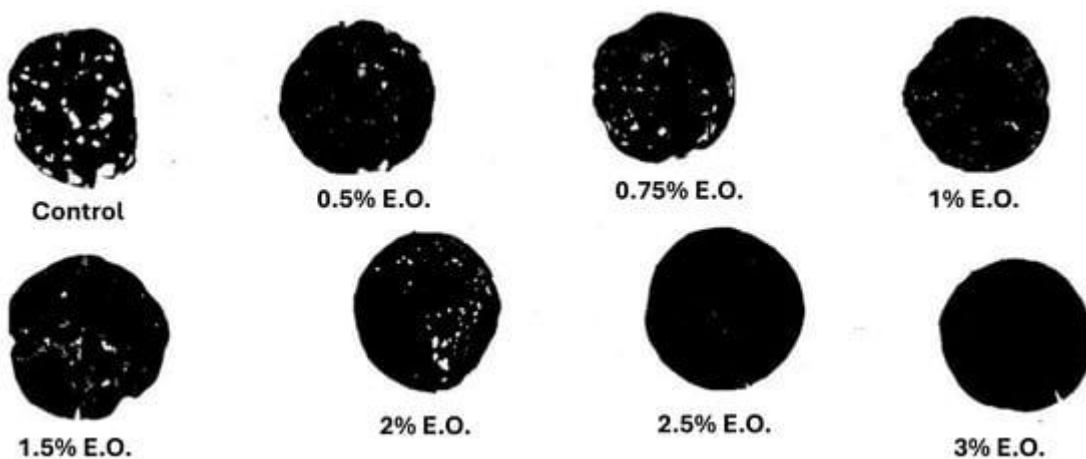


Figure 4. Example of eroded disc leaves at different doses of garlic EO processed with ImageJ v. 1.53 software.

3.3. *Effects of Garlic EO Nanoemulsion on Plants*

The results related to the plant growth exposed to the nano-formulation proved no statistical differences ($p > 0.05$ at all the sampling times) between the untreated control and the EO-treated plants (Figure 5). Indeed, all plants reached nearly the same height (about 30 cm). The recorded number of leaves per plant did not significantly vary across the different groups ($p > 0.05$ at all the sampling times) (Figure 6). Symptoms related to EO phytotoxicity appeared three days after treatment, and the number of symptomatic leaves continued to increase until 21 days after treatment, while the number of damaged leaves remained unchanged in subsequent assessments (Figure 7). In the treated plants, approximately four leaves per plant were damaged by the treatments, whereas in the untreated plants no leaves exhibited adverse symptoms throughout the experiment. The plants treated with the highest EO content (3%) displayed more severe symptoms ($P_i = 0.13 \pm 0.1$) compared to those treated with the lowest EO dose ($P_i = 0.09 \pm 0.01$).

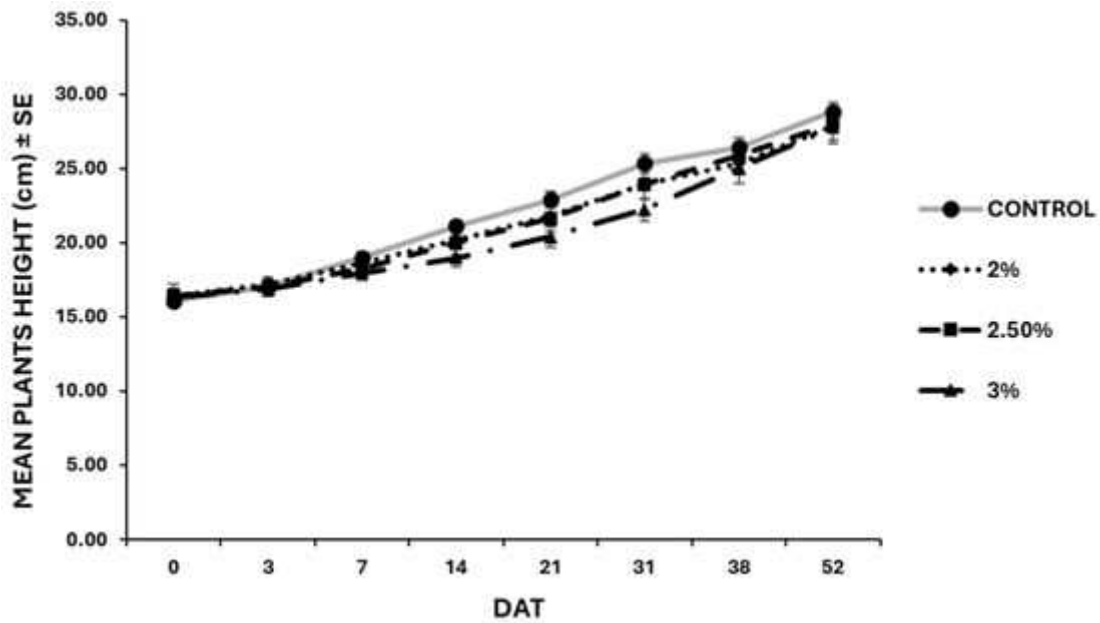


Figure 5. Plant height registered during the 52 days after treatment with garlic EO-based nano-formulation. No statistical differences ($p > 0.05$) were highlighted among the different treatments at the same time ($p > 0.05$). DAT = Days after treatment.

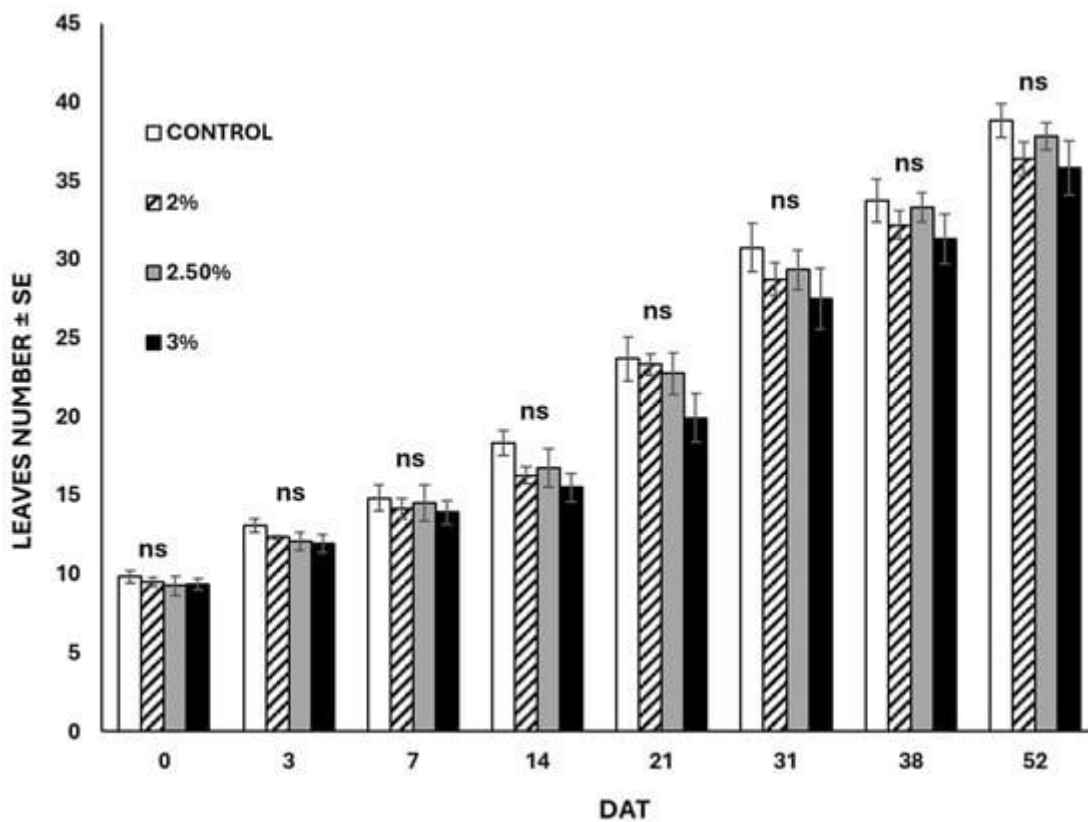


Figure 6. Number of leaves (mean \pm SE) recorded in plants treated with garlic EO-based nanoemulsion at different application rates. No statistical differences ($p > 0.05$) were highlighted among the different treatments at the same observation time. DAT = Days after treatment; ns = not significant.

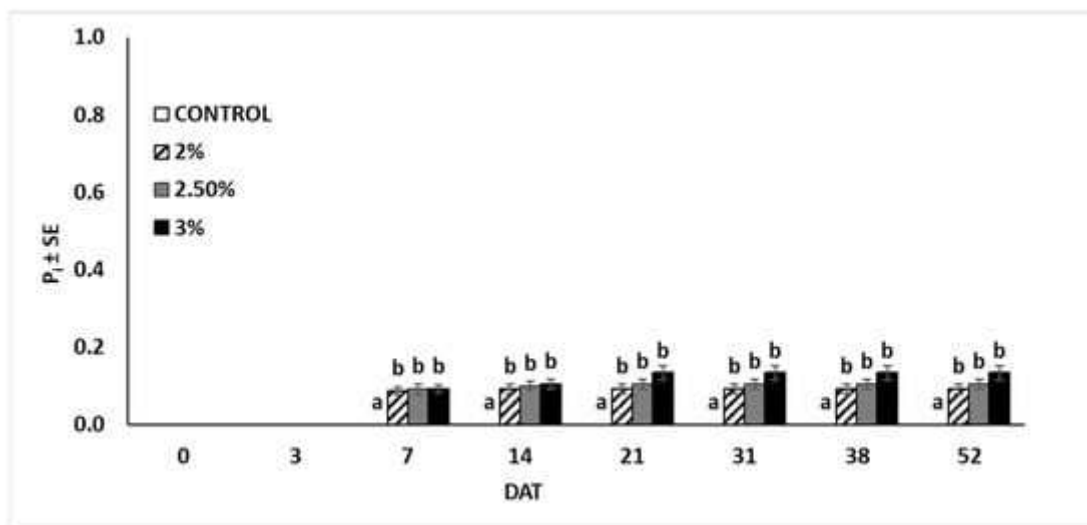


Figure 7. Phytotoxicity index (P_i) on pepper leaves caused by garlic EO nanoemulsion appeared 7 days after treatment. The P_i increased up to 21 days after treatment and remained constant until the end of the trial. (Means \pm SE) with different letters differ significantly according to Tukey's HSD post hoc test at $p \leq 0.05$. DAT = Days after treatment.

In terms of fruits produced by the plants, untreated plants yielded an average of 4.2 ± 0.4 fruits per plant, whereas EO-treated plants produced significantly fewer fruits ($F = 8.659$; $df = 3$; $p < 0.001$) because of the different application rates (Figure 8). At the end of the experiment (52 days after the treatments), no statistical differences ($F = 1.687$; $df = 3$; $p = 0.20$) were observed among the weights registered in the different treatments. Plants treated with 3 and 2.5% of EO reached a weight of 52.98 g and 49.82 g, respectively, whereas untreated plants weighed 47.22 g (Figure 9). Likewise, the roots of untreated plants were lighter compared to those of the treated plants, despite no statistical differences ($F = 2.290$; $df = 3$; $p > 0.05$) observed between treated and untreated plants (Figure 10). The chlorophyll content measured using the SPAD instrument decreased over time in both the treated

and untreated plants, although without significant differences ($F = 0.869$; $df = 3$; $p = 0.459$) (Figure 11).



Figure 8. Exponential increase in the number of pepper fruits in plants treated and untreated with garlic EO nano-formulation. Fruits appear in both groups of plants 31 days after treatment. (Means \pm SE) with different letters differ significantly according to Tukey's HSD post hoc test at $p \leq 0.05$. DAT = Days after treatment; ns = not significant.

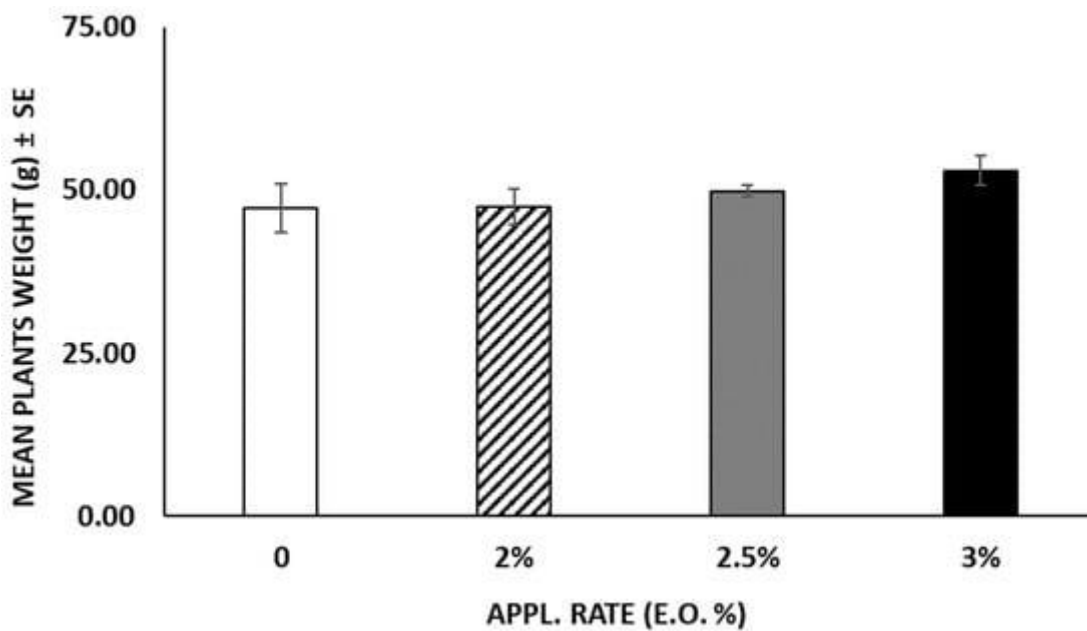


Figure 9. Mean plant weight (mean \pm SE) was obtained at the end of the experiment after cutting and separating the roots into the soil. No statistical differences ($p > 0.05$) were highlighted among the different treatments. APPL. RATE = application rate.

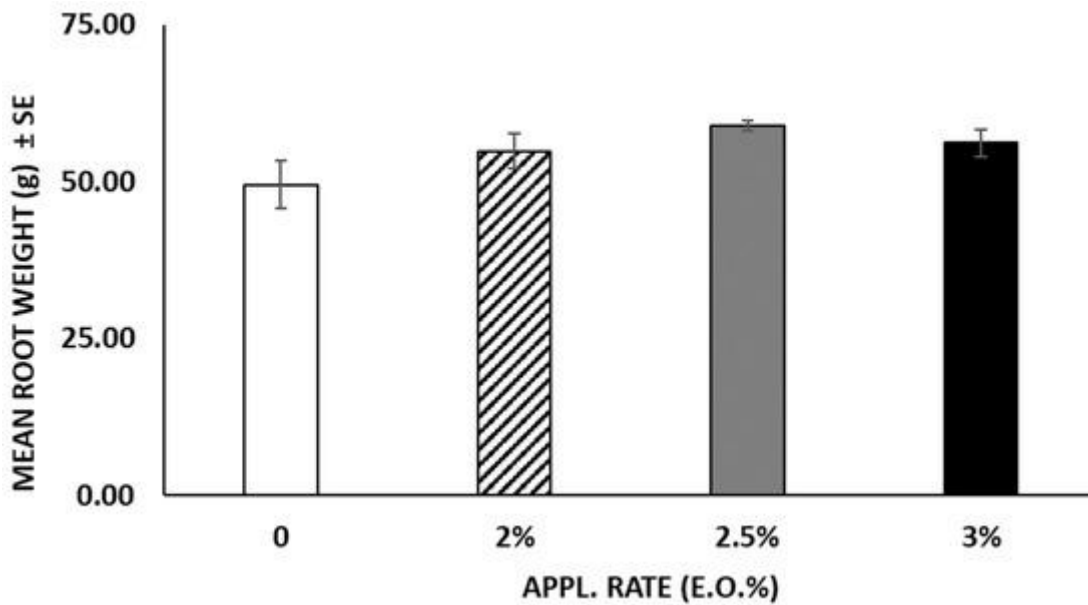


Figure 10. Mean root weight (mean \pm SE) was obtained at the end of the experiment after cutting the plants. No statistical differences ($p > 0.05$) were highlighted among the different treatments.

APPL. RATE = application rate.

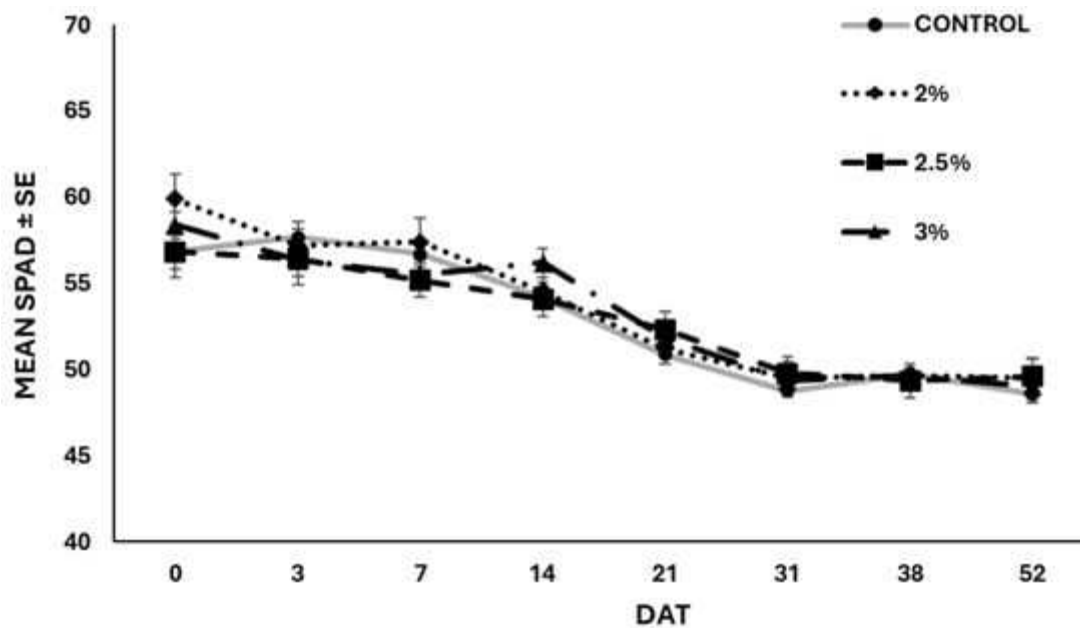


Figure 11. Decrease in chlorophyll inside the leaves (mean \pm SE), measured by the SPAD optical sensor. No statistical differences ($p > 0.05$) were highlighted among the different treatments (ANOVA, Tuckey's HSD post hoc test). DAT = Days after treatment.

4. Discussion

The main objective of this study was to develop an *A. sativum* EO-based nanoemulsion characterized by a high concentration of the active ingredient (15%) for the control of the key horticultural pest, *S. littoralis*. Subsequently, we evaluated the potential phytotoxic effects of garlic EO nano-formulation on bell pepper plants under greenhouse conditions.

The physical characteristics of the developed insecticidal formulation indicated a good quality both in terms of size and PDI, as well as in terms of stability. These parameters are essential for assessing the quality of the nanoemulsion and predicting its stability over time [24]. Nanoemulsions, possessing small droplet sizes (<200 nm), are considered favorable for their application against insect pests. Furthermore, the low PDI value suggests a highly homogeneous emulsion, which is less susceptible to the destabilization phenomenon known as Ostwald ripening [25]. The EO formulation proposed in this study had a mean particle size below 141 nm and a PDI near zero, confirming its good quality. Similar results were obtained by Ricupero et al. [26], who developed a nanoemulsion of garlic EO using the same self-emulsification process combined with sonication. Indeed, these authors obtained a slightly larger droplet size (176.23 ± 0.9 nm) and a less-homogeneous formulation (PDI = 0.18) compared to the one obtained during this research (PDI = 0.146). Since the methodology and procedure were the same, the differences between the two nanoemulsions, the present one and that produced by Ricupero et al. [26], can be attributable to the different batches of garlic EO used. On the other hand, Modafferi et al. [27], using a similar procedure, obtained particle size, PDI, and zeta potential comparable to those obtained in this study (size = 159 nm; PDI = 0.18; zeta potential = -21.9 mV). This evidence suggests that the EO composition is key in formulating EO-based nano-delivery systems.

Variability in the composition of EOs is common. It can depend on various factors, such as the geographical origin, the season of growth and harvesting, and the cultivar or variety used [28,29]. As an example, perillaldehyde was the main constituent of the oil of *ippia javanica* var. *javanica* (Verbenaceae). At the same time, myrcenone (ipsdienone) was the main compound in the oils of *L. javanica* var. *whytei* [30]. In addition, the same authors highlighted the fact that while perillaldehyde, linalool, and carvone, components of the oil of *L. javanica* var. *javanica*, were toxic to adult *Sitophilus zeamais* (Motschulsky) (Coleoptera: Curculionidae), myrcenone, the other main component of the oil, was not; thus, plant variety can impact the EO insecticidal activity against target pests. The same authors also observed that EO yield and chemical composition varied significantly with harvest time during the season, for both varieties.

The physical properties of a nano-formulation can also be affected by the other ingredients and co-formulants used for its production. For example, a high relative amount of surfactant ensures the production of tiny droplets with an optimal size distribution (i.e., PDI tending towards zero). Nevertheless, the amount of surfactant should be reduced as much as possible when developing insecticides for crop protection, as suggested by Modafferi et al. [23], because of their phytotoxicity. In addition, the quality parameters (i.e., size, PDI, and particle surface charge) are strongly influenced by the approach used to prepare the nanoemulsion, as reported by Modafferi et al. [23], who used the same oil batch but had a different preparation process (i.e., high-pressure fluidization).

Regarding the insecticidal efficacy of the *A. sativum* EO-based nano-formulation, our findings suggest its potential for the control of *S. littoralis*. Indeed, almost 100% mortality of larvae exposed to the treatments was achieved at the two highest-tested concentrations. The efficacy of garlic EO against crop pests has already been assessed against other insect pest groups, such as Hemiptera [23], Lepidoptera [26,31], Isoptera [32], Coleoptera ([33,34]), Mallophaga [35], and Diptera [36], as well

as against other arthropods, such as Acari [16]. On the other hand, few studies on garlic EO or extracts applied as insecticides against *S. littoralis*. Ali et al. [37] tested garlic EO aqueous solutions using Triton X-100 as an emulsifier for evaluating mortality and anti-feeding activity against *S. littoralis* fourth instar larvae. The authors highlighted the LC₅₀ and LC₉₀ values of 19.95% and 39.18% (w/w) of garlic EO, respectively, while the LC₅₀ and LC₉₀ values for lemon EO were higher (24.20% and 47.07%, respectively). Furthermore, garlic EO at a low concentration (LC₃₀ = 16.30% of EO) revealed antifeedant activity with a reduced castor leaf consumption ranging from 62% to 74%, depending on the time post-treatment [37]. Our results showed significant antifeedant effects of the garlic EO nano-formulation, which varied depending on the application rates. However, at the maximum application rate (3% of EO), the exposed larvae did not feed, while at the lowest dose (0.5% of EO), only 30% of the disc leaves were consumed by larvae. Our results suggested that garlic EO nanoemulsion highly improved the bioactivity of crude EO, by reducing the amount of EO needed to kill or deter *S. littoralis* larvae. Thus, nano-formulation could have practical application in the field, while the amount required for pure EO could impair its use. In addition, EO-based nanoemulsion guarantees high stability and long persistence in the field, proving effective bioactivity and pest control for at least 72 h after plant treatment.

Apart from garlic EO, other EOs revealed promising insecticide and antifeedant activity against *S. littoralis* (reviewed in [38]). As an example, *Artemisia monosperma* (Asteraceae), *Callistemon viminalis* (Myrtaceae), *Citrus aurantifolia* (Rutaceae), and *Cupressus macrocarpa* (Cupressaceae) exhibited good insecticidal activities, affecting larval growth and feeding ability of fourth instar larvae, depending on the doses used in the trials [39]. From the literature, it has been highlighted that the insecticidal activity of EOs was tested exclusively under laboratory conditions against *S. littoralis*, and almost one-third of the research involved Lamiaceae EOs. Nevertheless, several

biological activities were investigated (i.e., repellence and deterrence, larvicidal, ovicidal, and biochemical effects, and growth regulation), although most substances acted as antifeedants and contact insecticides [38]. Nevertheless, most of the articles reviewed by Jbilou et al. [38] tested EO bioactivity at less than 48 h only on third- and fourth-instar larvae. Here, we tested the mortality until 72 h, and we selected second-instar larvae, which is considered the best-targeted stage for effective pest control.

To the best of our knowledge, the negative effects of *A. sativum* EO used as a pesticide on bell pepper plants (i.e., phytotoxicity) have not been previously investigated. Nevertheless, a garlic EO-based nano-formulation had a negligible phytotoxic effect on tomato plants for up to 14 days after the insecticide treatment against *T. absoluta* [26]. In contrast, our results indicated plant damage beginning a few days after treatment. In pepper plants treated with the highest dose (3% of EO), leaf damage was observed in 8% of the plants just three days after treatment. This percentage increased to 13% by 21 days after treatment, resulting in approximately four damaged leaves per plant. Furthermore, significant differences were observed in the number of fruits produced among plants treated with different amounts of garlic nanoemulsion compared to untreated ones, suggesting a negative impact on fruit setting. These findings underscore the need for further investigations into the potential non-target effects of *A. sativum* EO on plant health and highlight the importance of careful consideration in pesticide application to minimize unintended harm to crops.

5. Conclusions

The quality of the garlic EO nanoemulsion was promising, and the evaluated physical parameters indicated good stability and potential efficacy against the target pest. Indeed, a good larvicidal activity of the EO formulation was recorded, suggesting its possible application in biological and/or integrated control programs to control *S. littoralis* larvae. Garlic EO-based nanoemulsion induced significant

mortality in the target pest and, contextually, markedly reduced larval feeding activity in plants. Despite observing phytotoxicity symptoms in pepper plants when treated with the garlic EO nanoemulsion, these plants successfully completed their life cycle and produced fruit with slightly different outcomes than those of untreated plants. This aspect should be further investigated to evaluate interferences of garlic EO and its formulations for crop plants.

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IV. Exploring garlic nano-emulsions for *Tuta absoluta* control, their impact on the intestinal microbiome and their side effects on *Nesidiocoris tenuis*

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Abstract

The South American tomato pinworm, *Tuta absoluta* Meyrick, is a major destructive pest on tomato crops. Currently, the management of this pest in both field and greenhouse settings relies mainly on synthetic insecticides. However, concerns regarding the indiscriminate use of these insecticides, coupled with the growing demand for safer and more eco-friendly solutions, have driven researchers to explore alternative pest control strategies. Among the potential options, essential oils (EO) have shown great promise, with nanoemulsions (NE) further enhancing their stability and effectiveness. The main goal of this study was to develop garlic nanoemulsions with a high active ingredient concentration and a low surfactant percentage, ensuring long-term stability and small particle size. This was accomplished using both low (LP)- and high (HP)-pressure microfluidization techniques. The LP nano-emulsion was produced at 2,000 psi, while the HP nano-emulsion was achieved at 20,000 psi. The synthesized nanoemulsions were tested in laboratory conditions to evaluate their toxic effects on *T. absoluta* larvae, their impact on the predator *Nesidiocoris tenuis* Reuter, and their potential phytotoxicity on tomato plants. Both nanoemulsions exhibited a low polydispersity index (PDI) with high stability over time. The two nanoformulations demonstrated significant toxicity against second-instar larvae of *T. absoluta* 72 hours after treatment, with LD₉₀ values of 0.886 g/L and 1.062 g/L for the LP and HP nanoformulation, respectively. In addition, 100% mortality was observed in larvae exposed to the highest tested concentration. In contrast, *N. tenuis* adult mortality at 72 hours was less than 20% for both nanoemulsions. In phytotoxicity tests, the HP nanoemulsion exhibited low levels of phytotoxicity, while the LP nanoemulsion damaged tomato plants (33%) at the LD₉₀ concentration. Additionally, this study explored for the first time the intestinal microbiome of *T.*

absoluta larvae exposed to garlic nanoemulsions, revealing significant differences between treated and untreated specimens, suggesting a direct effect of the EO treatments on the bacterial communities. The results of this study indicate that garlic nanoemulsions could be an effective option for managing *T. absoluta* populations in the field. However, further research is needed to develop and test formulations that ensure minimal phytotoxicity.

Keywords: microfluidization, phytotoxicity, polydispersity index (PDI), toxicity, stability

1. Introduction

Tuta absoluta Meyrick (Lepidoptera: Gelechiidae), commonly known as the South American tomato pinworm or tomato borer, is one of the most destructive pests affecting tomato crops, with its origin traced to South America (Desneux et al., 2011; Urbaneja et al., 2011). Although it was originally confined to this continent (Guillemaud et al., 2015; Biondi et al., 2018), its presence was reported in Spain in 2006 (Urbaneja et al., 2007) and it is now a global threat (Biondi et al., 2018).

The larvae of *T. absoluta* can damage all parts of a tomato plant, including leaves, flowers, stems, and fruits (Urbaneja et al., 2011). Pest infestation and the subsequent development of pathogens can result in severe crop losses, reaching 80–100% depending on the infestation severity (Desneux et al., 2011; Cuthbertson et al., 2013). This moth is a key pest for tomato crops in greenhouse and open-field conditions, primarily due to its ability to complete up to 13 generations per year (Tropea Garzia et al., 2012). Currently, the most widely used strategy for controlling this pest in both environments relies on the use of synthetic insecticides (Guedes and Picanço, 2012; Guedes and Siqueira, 2012; Campos et al., 2017; Biondi et al., 2018; Desneux et al., 2022), despite

the growing emphasis on integrated pest management (IPM) in national and international guidelines. The widespread and indiscriminate use of these chemicals has contributed to the development of insecticide resistance in *T. absoluta* populations (Roditakis et al., 2017; Haddi et al., 2012) and has also adversely affected non-target organisms, such as predators and pollinators (Biondi et al., 2012; 2013; Ahmed et al., 2024). In response to these challenges and the increasing consumer demand for pesticide-free food, there is growing interest in alternative, more sustainable control methods. Bioinsecticides, including Spinosad, *Beauveria bassiana*, *Bacillus thuringiensis*, and azadirachtin, have gained prominence in IPM as environmentally friendly solutions (Silva et al., 2011; González-Cabrera et al., 2011; Biondi et al., 2018). These alternative control tools aim to reduce the environmental impact of pest control while maintaining effective management of *T. absoluta* populations (Giunti et al., 2019).

Plant extracts, particularly essential oils (EOs), are appealing options among the potential alternatives. They are widely available and accessible, and numerous studies have demonstrated their insecticidal effectiveness against various insect pests while showing low toxicity to humans (Isman et al., 2020a, b; Raveau et al., 2020). When used correctly, EOs can provide a sustainable and ecological approach to pest management, aligning with the principles of IPM and organic farming practices. Additionally, EOs can exhibit broad-spectrum activity due to their complex chemical compositions, which feature multiple bioactive compounds. This complexity reduces the chances of developing resistance in insects (Yuan et al., 2018). Furthermore, most of the EOs tested as insecticides have a long history as food and cosmetics ingredients, making them widely accepted by consumers as safe for human health and the environment.

Several studies have demonstrated the insecticidal efficacy of formulations based on EOs or botanical extracts for controlling pests in horticultural crops, including lepidopteran species such as *T. absoluta* (Campolo et al., 2017; Chegini and Abbasipour, 2017; Piri et al. 2020; Ricupero et

al., 2022; Ben Abdallah et al., 2023). Despite their effectiveness, EOs face several challenges, such as low stability, rapid degradation, limited persistence, and potential phytotoxicity, which can be mitigated or minimized through appropriate formulation techniques. These limitations can be addressed by incorporating nanotechnology into EO formulations. For instance, high-energy emulsification techniques, such as microfluidization, have produced stable nanoemulsions with enhanced bioavailability and insecticidal activity (Modafferi et al., 2024). These nanoformulations improve the persistence and efficacy of essential oils and reduce their environmental impact, making them promising candidates for inclusion in integrated pest management programs (Modafferi et al., 2024b).

In recent years, the study of the gut microbiome of agricultural pests has become a crucial tool for understanding the interaction between insects and insecticide treatments (Peterson, 2024). The microbiome has been shown to play a significant role in insecticide resistance, digestion of toxic compounds, and adaptation to environmental changes (Peterson, 2024). However, the impact of new control strategies, including essential oil-based nanoemulsions, on the gut microbiome of pests like *T. absoluta* remains an underexplored area. Alterations in the gut microbiota could directly affect the efficacy of treatments by modifying the insect's ability to metabolize toxic compounds or altering its overall physiology (Peterson, 2024). Given that garlic nanoemulsions have proven highly effective against *T. absoluta*, it is crucial to investigate how these treatments affect the intestinal microbiota of exposed larvae. This could provide a more comprehensive understanding of the mechanisms underlying pest resistance and susceptibility to these bioinsecticides, opening new avenues for optimizing integrated pest management (IPM) approaches through microbiome manipulation.

This study aimed to develop two stable garlic (*Allium sativum* L.) EO-based nano-emulsions using the high- and low-pressure micro-fluidization techniques. The objective was to create

formulations with a high active ingredient concentration (i.e., 15%) and a low surfactant content while maintaining long-term stability. In addition to testing these nano-formulations against *T. absoluta* larvae, the study also aimed to evaluate their sub-lethal on the predatory mirid *Nesidiocoris tenuis* Reuter (Hemiptera: Miridae), a key natural enemy of *T. absoluta* (Pérez-Hedo and Urbaneja, 2016), as well as their potential phytotoxicity on tomato plants. Lastly, this study explored the intestinal microbiome of *T. absoluta* larvae exposed to garlic nanoemulsions for the first time.

2. Materials and method

2.1. Insects and plants

The *T. absoluta* strain was maintained at the Entomology Laboratory of the Instituto Valenciano de Investigaciones Agrarias (IVIA), following the methodology described by Campolo et al. (2017). Larvae were reared on tomato plants, while adults were sustained with a sugar solution. Both adults and larvae were kept in ventilated insect-rearing cages measuring 63 x 24.5 x 24.5 cm (BugDorm-1 Insect Tents; MegaView Science Co., Ltd., Taichung, Taiwan).

Nesidiocoris tenuis adults were purchased from Bioline AgroSciences (Essex, UK) and reared under laboratory conditions using green bean pods and *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) eggs as food. For the bioassays, F1 generation adults were used. Adult specimens were collected with a mechanical aspirator, sexed, and grouped into batches of 5 females and 5 males, placed in ventilated 50 ml plastic tubes (Falcon®; Corning, Glendale, United States) before being used in the EO trials. All experiments used tomato plants (*Solanum lycopersicum* L. cv Moneymaker). The tomato plants were grown from untreated seeds in 8 x 8 x 8 cm pots filled with organic soil. Plants were regularly

watered throughout the growth period. Both insects and tomato plants were kept under controlled conditions ($25^{\circ} \pm 2$, $50\% \pm 10$ relative humidity, and a 14:10 (L:D) photoperiod).

2.2. Development and characterization of Garlic EO-based (GEO) nano-formulation

The *A. sativum* EO-based nanoemulsions were developed using a high-energy emulsification process described by Modafferi et al. (2024). Initially, the raw emulsion was created using the self-emulsification method, where GEO and Tween 80® (polyoxyethylene (20) sorbitan monooleate) (Sigma- Aldrich, Munich, Germany) were mixed in a 3:1 (w:w) ratio for 30 min at 7,000 rpm at $25 \pm 2^{\circ}$ C. Double-distilled water was gradually added (1 mL min^{-1}) in a 4:1 (w:w) ratio to the emulsion without further mixing. The resulting emulsion (GEO 15% w/w; Tween 80® 5% w/w; water 80% w/w) was stirred at 7,000 rpm for 3 h. Subsequently, aliquots of the raw emulsion were homogenized using a high-pressure microfluidizer (LM20 Microfluidizer™ Processor, USA) for 5 cycles at two different pressures: 20,000 (high-pressure, HP formulation) and 2,000 (low-pressure, LP formulation) PSI.

2.3. Laboratory bioassay on *Tuta absoluta* larvae

The insecticidal activity of the GEO nanoemulsions was tested on second-instar *T. absoluta* larvae. Experiments were conducted under controlled environmental conditions ($25 \pm 2^{\circ}$ C, $60 \pm 10\%$ RH, 14:10 L:D). Seven GEO concentrations (0.25, 0.375, 0.5, 0.75, 1, 2, and 2.5%) were tested for each formulation, and water + Tween 80® was used as a negative control treatment. These concentrations were selected based on preliminary observations to achieve 10% and 90% mortality rates.

The leaf-dip method was employed to apply the GEO formulations. Tomato leaflets were dipped in each formulation for 10 seconds and air-dried for 45 minutes. Once dried, the leaflets were placed

individually in a sterile plastic arena (5.5 x 3.5 cm) containing a wet cotton wool ball to guarantee humidity. Afterward, a single second-instar larva was gently introduced into each arena using a fine paintbrush. The arenas were covered with a mesh to prevent larvae from escaping and ensure adequate ventilation while avoiding fumigation. Thirty replicates were performed for each concentration and control. Mortality was assessed daily for three days, and larvae were considered dead if they showed no movement when gently stimulated with a fine brush.

2.4. Evaluation of GEO formulations on *Nesidiocoris tenuis*

The potential non-target effects of the GEO nanoemulsions on *N. tenuis* adults (males and females) were evaluated using the LD50 and LD90 values previously obtained for *T. absoluta* larvae. Experiments were conducted under controlled environmental conditions ($25 \pm 2^\circ\text{C}$, $60 \pm 10\%$ RH, 14:10 L:D). Two exposure routes were tested: topical and ingestion.

In topical application, each *N. tenuis* adult was treated with 3 μl of the respective EO-based formulation applied to the abdomen using a micropipette for topical treatments. Control treatments included a negative control water + Tween 80 and a positive control using acetamiprid at the maximum recommended rate (3 mL/L). Before treatment, adults were sexed and grouped into batches of 10 males and 10 females. To immobilize the insects for a more straightforward application of the formulations, specimens were placed in an insulated thermal box with dry ice for 3 minutes (Passos et al., 2022). Three replicates were conducted for each concentration, including 20 individuals (10 males and 10 females) per replicate. Mortality was recorded daily for three days, and insects were considered dead if they exhibited no movement upon stimulation with a fine brush.

In ingestion trials, each concentration, along with the untreated (water + Tween80) and positive controls (acetamiprid at the maximum labeled rate), was replicated 8 times for the ingestion method.

In each replicate, 10 *N. tenuis* adults (5 pairs) were exposed to treated plants. Tomato plants were sprayed with garlic nanoemulsions, and after air drying, two treated tomato plants were placed in insect-rearing cages measuring 63 x 24,5 x 24,5 cm (BugDorm-1 Insect Tents). Then, 10 *N. tenuis* adults were released into each cage. Mortality was assessed daily for three days, with insects considered dead if they did not respond to gentle stimulation with a fine brush.

2.5. Phytotoxic effects of garlic nano-formulations towards tomato plants

A specific experiment was designed to evaluate the potential non-target phytotoxic effects of the two nanoformulations on tomato plants. The experiment assessed the effects of both GEO (LP and HP) formulations at their respective LD₃₀, LD₅₀, and LD₉₀ values as estimated for *T. absoluta*.

Tomato plants were sprayed with the two nanoformulations until runoff and subsequently were kept under controlled environmental conditions in growth chambers (25± 2 °C, 60± 10% RH, 14:10 L:D) until the end of the experiment. Each concentration, including the untreated controls (water and water + Tween 80), was replicated six times. Phytotoxicity was assessed using the phytotoxicity index (P_i) as described by Campolo et al. (2017), which was calculated as follows:

$$P_i = \sum_{j=0}^n \left(\frac{DL_j}{TL} \times \frac{DC}{n-1} \right)$$

where DL is the number of damaged leaves in each damage severity class j , TL is the total number of leaves sprayed, DC is the damage severity class, and n is the number of damage severity classes (0 = no phytotoxicity damage; 1 = 25% of damaged leaf surface; 2 = 50% damaged leaf surface; 3 = 75% damaged leaf surface; 4 = 100% of damaged leaf surface). The P_i values range from 0 (no damage) to 1 (dead leaves). Leaf damage observations were recorded at 24h, 48h and 7 days after treatment.

2.6. Intestinal microbiome on *T. absoluta* larvae

The impact of GEO nanoemulsions on the bacterial communities of *T. absoluta* was evaluated on second instar larvae. The specimens were exposed to tomato plants treated with LD₃₀ and LD₇₀ concentrations of the nanoemulsion at HP (0.22% and 0.51 %, respectively) and were collected after 24h and 72h. Larvae that survived the treatments were individually placed into 1.5 µl Eppendorf tubes and stored at -80°C. Three replicates were made for each concentration (LD₃₀ and LD₇₀), including negative controls (water and water + Tween 80®). Each replicate consisted of a pool of five *T. absoluta* larvae, resulting in a total of 15 larvae per concentration at both 24h and 72h exposure time. Before molecular analysis, the larvae were surface sterilized by immersing them in 0.1% sodium hypochlorite for 1 minute, followed by rinsing with sterile water. Total DNA was extracted using the NZY Tissue Gdna isolation kit (Nzytech) kit following the manufacturer's protocol. Bacterial communities were characterized through the amplification of the hypervariable region V3 and V4 using primers 341F (CCTACGGGNGGCWGCAG) and - 805R (GACTACHVGGGTATCTAATC). Amplicon library preparation was performed according to recommended protocols (Illumina Demonstrated Protocol: 16S Metagenomic Sequencing Library Preparation) and then sequenced on an Illumina Miseq by Macrogen (Netherlands). The raw 16S rRNA gene amplicon data were analyzed using the DADA2 R package (Callahan et al., 2016) in RStudio (version 4.2.2). Following the inspection of read quality profiles, low-quality reads were filtered and trimmed. The remaining sequences were merged, dereplicated, and classified into unique Amplicon Sequence Variants (ASVs). ASV clustering with 100% sequence identity was preferred over operational taxonomic unit (OTU) clustering, which generally represents clusters with less than 100% sequence identity, due to its superior reusability, reproducibility, and resolution (Callahan et al., 2017). Taxonomic classification of the ASVs was performed using a classify-by-consensus approach, with Silva SSU

(v. 138.1) (Quast et al., 2012) as the reference database. The ASVs identified as mitochondria or chloroplasts were removed from the dataset. The obtained read-counts were then normalized using the Cumulative Sum Scaling (CSS) method.

The normalized feature table was analyzed following the methodology outlined in Maretto et al. (2023). Specifically, the MicrobiotaProcess package (Xu et al., 2023) was employed to perform alpha- and beta-diversity analyses and to identify the key taxa comprising the core microbiome. Alpha diversity, which assesses within-sample diversity, was evaluated using the Chao1, Shannon, and Simpson 1-D indices. Beta diversity, which examines variations in microbial composition between samples, was assessed through Principal Coordinate Analysis (PCoA) based on the Bray-Curtis dissimilarity matrix. Additionally, statistical analyses, including PERMANOVA and PERMDISP2, were conducted to further explore and quantify differences in beta diversity. Venn diagrams were generated to underline shared ASVs between treated and untreated samples of *T. absoluta*. All plots were generated using ggplot2 R-package (Wickham 2016).

The sequencing data are available in the NCBI database under project number PRJNA1197432

2.7. Data analysis

The mortality data for *T. absoluta* and *N. tenuis*, and the plant damages were analyzed using a Univariate Analysis of Variance (ANOVA) and an HSD Tukey post-hoc test to determine significant differences between treatments. Mortality observed in EO-treated insects was calculated using the Abbot formula. Probit analysis (Finney, 1947) was used to estimate the LD₃₀, LD₅₀ and LD₉₀; values, with significance determined by the absence of overlap in the 95% fiducial limits. The ANOVA assumptions of normality and homoscedasticity of variance were satisfied for all datasets ($P > 0.05$). Statistical analysis was performed using IBM SPSS Statistics version 19.

3. Results

3.1. GEO nanoemulsions physical characteristics

The physical characterization of developed formulations is reported in Table 1. The relatively low polydispersity index (PDI) suggests a narrow size distribution and uniformity. In addition, the nanoemulsion exhibited a negative charge on the particle surfaces. During the experiments no evidence of phase separation of the developed nano-insecticide was observed, confirming the stability of the nanoemulsion systems.

3.2. Insecticidal activity on *T. absoluta*

The two developed GEO formulations exhibited high toxicity against second-instar larvae of *T. absoluta*. During the entire observation period (i.e., 72h), no mortality was recorded in the untreated controls (water and water + Tween80). The mortalities induced by the LP nanoformulation were significantly different among the application rates used ($F=114.508$; $df=8$; $P<0.01$) (Figure 1). The time after the treatments also influenced the number of dead larvae ($F=4.265$; $df=2$; $P<0.05$).

The mortality data fitted the probit model (Table 2). The different LD values showed that the insecticidal efficacy of the LP GEO formulation occurred mainly during the first 24 hours after treatment. The amounts of EO required to kill 50 and 90% of the exposed larvae at 24 h were 0.489 and 1.449 %, respectively, whereas 48h after treatment, the amounts were 0.394 and 1.190 %.

Larval mortalities caused by HP nanoformulation also showed significant differences due to the application rates ($F=111.425$; $df=8$; $P<0.001$), with mortality rates significantly increasing over time ($F=3.102$; $df=2$; $P<0.05$) (Figure 2). The mortality data related to HP nanoformulation also fitted the probit model (Table 2.), with amounts of EO required to kill 50 and 90% of the larvae at 24 and 48 h were 0.496 and 1.419 %, and 0.420 and 1.263 %, respectively.

3.3. Lethal effect on *N. tenuis*

The mortality induced by the nanoformulations administered via the ingestion route to *N. tenuis* showed statistical differences between the positive control (Acetamiprid) and the other treatments ($F = 383.968$; $df = 5$; $P < 0.001$). Time after the treatments also influenced the number of dead adults ($F = 56.921$; $df = 2$; $P < 0.001$). Twenty-four hours after treatment, a mortality rate of 50% was recorded in *N. tenuis* adults exposed to acetamiprid-treated plants, while on plants treated with the two GEO nanoemulsions, the mortality rate was close to nihil. Furthermore, 72 h after treatment, 86% of adults exposed to acetamiprid-treated plants died. In contrast, only 16% and 12.5% mortality were recorded for the garlic nanoemulsions for LP and HP formulations, respectively, at the LD₉₀ (Figure 3).

The topical treatment on *N. tenuis* adults also highlighted statistical differences between acetamiprid and the LDs of the two nanoemulsions ($F = 254.326$; $df = 5$; $P < 0.001$). Time after treatment influenced adult mortality ($F = 84.842$; $df = 2$; $P < 0.001$). Twenty-four hours after treatment, a mortality rate of 27% was recorded in acetamiprid-treated adults, while no mortality was observed with the two garlic nano-emulsions. In addition, 72 h after treatment, 60% mortality was recorded in positive control, compared to 11.7% and 16.3 % mortality for LD₉₀ LP and HP-treated adults, respectively (Figure 4).

3.4. Phytotoxic effects of garlic EO-based nano-formulations on tomato plants

Phytotoxic symptoms due to the different application rates of the HP nanoemulsion began to appear 48h after treatment, while in plants treated with the LP nanoemulsion, symptoms appeared after 24h. For the HP nanoformulation, the results showed no statistical ($P > 0.05$) phytotoxicity between untreated control plants and the GEO-treated plants ($F = 1.103$; $df = 4$; $P = 0.377$) (Figure 5). In

plants treated with GEO-HP formulation at LD₃₀, 8.33% damage was observed, while at LD₅₀ and LD₉₀, 12.5% damage was noted starting 7 days after treatment (Figure 6). In contrast, plants treated with LP nanoformulation showed significantly higher phytotoxicity than controls ($F = 12.000$; $df = 4$; $P < 0.001$). Specifically, plants treated with LD₃₀ exhibited 25% damage. After 7 days, plants treated with LD₅₀ and LD₉₀ showed 33% damage in both treatments. In plants treated with water and Tween 80, phytotoxicity rates of 8.33% (HP formulation) and 13% (LP formulation) were observed after 7 days, while no damage was recorded in the water-treated control plants.

3.5. Intestinal microbiome on T. absoluta larvae

Sequencing on the Illumina MiSeq Platform of the 24 field samples, consisting in pools of 5 individuals according to different Treatments (EOs LD₃₀, EOs LD₇₀, Control H₂O, and H₂O+ Tween) and Time (24h and 72h), yielded 5 757 294 raw reads. After quality filtering, denoising, and dechimerising 5 171 743 reads were obtained with an average length of 328 nucleotides. One hundred and twenty-two ASVs were counted after normalizing and discarding those corresponding to eukaryotic DNA and the endosymbiont Wolbachia.

Bacterial community structure in EO treated and untreated samples

The bacterial microbiome of samples collected after 24h of the treatment was represented by 14 phyla while samples collected after 72h showed 9 phyla. In both cases Proteobacteria (76% and 97%, respectively) and Bacteroidota (11% and 1.7%, respectively) were predominant (Supplementary material Fig. S1). After 24h, the microbiota of the control samples (water and water + Tween) was dominated by bacteria of the family Enterobacteriaceae followed by

Sphingomonadaceae in the water and Comamonadaceae for water plus Tween. Treatment samples were dominated by Comamonadaceae and Pseudomonadaceae for LD₃₀, and LD₇₀ respectively.

The bacterial communities after 72h were predominantly represented by Enterobacteriaceae followed by Moraxellaceae in the water and Weeksellaceae and Pseudomonadaceae for water plus Tween. The families Pseudomonadaceae and Sphingomonadaceae were the most predominant at LD₃₀ and LD₇₀ respectively.

Comparisons of bacterial diversity in EO treated and untreated samples

Observed ASVs' richness and homogeneity calculated using Chao1, Shannon and Simpson 1-D indices showed no significant differences after 24 or 72 hours of treatments. However, a distinct pattern emerged between treated and untreated samples, particularly after 24h, with higher richness and diversity observed in samples treated with water or water + Tween compared to those exposed to the EO treatments (Supplementary material Fig. S1).

Beta diversity measures showed that bacterial communities did not differ significantly among treatments after 24h while a significant difference was present among treatments after 72h. In particular, after 72h the Permutational Multivariate Analysis of Variance (PERMANOVA) returned to a p-value = 0.001 for 999 permutations, and the Analysis of Similarities (ANOSIM) reported an R value = 0.5617 and a p-value = 0.002. In addition, both the PCA and the PCoA showed some dissimilarities among treated and untreated samples after 72h (Supplementary material Fig. S3). The control group exhibits a more diffuse cluster, suggesting the presence of multiple main components shaping the characteristics of the microbiota. In contrast, the treated group displays a more circumscribed and well-defined cluster.

Furthermore, Alpha and Beta diversity were calculated separately for each treatment (Control, Control Tween, LD₃₀, and LD₇₀) to assess differences of the bacterial communities at 24 and 72 hours. No significant differences were observed for any of the treatments. DESeq2 was also used to analyze the differential bacteria within *T. absouta* larvae after different treatments. Two ASVs corresponding to the genus *Staphylococcus* and *Blastomonas* were identified as significantly different among treatments after 72h. *Staphylococcus* sp. was detected in treated samples but was absent in control samples (Water) and sparsely represented in samples treated with water + Tween. Similarly, *Blastomonas* sp. was present in samples treated with both EO doses but completely absent in untreated samples.

Twenty-four hours after the treatments, the Venn diagram showed 9.8% of shared ASVs (n=10) among treated and untreated samples and a similar percentage of specific ASVs (i.e., 23%, 15%, 15% and 13.5% for water, water + Tween, LD₃₀ and LD₇₀ respectively) (Supplementary material Fig. S4). A percentage of 12.5% shared ASVs was observed after 72h. Samples treated with water + Tween exhibited a notably higher percentage of specific ASVs (28.75%) compared to the other treatments, which showed 7.5% (Water), 10% (LD₃₀), and 13.75% (LD₇₀), respectively.

The interaction between treatments and time was also evaluated. While alpha diversity indices revealed no significant differences among samples, beta diversity measures indicated that bacterial communities across the entire dataset differed significantly when considering the overall data (PERMANOVA $p=0.002$; ANOSIM $p=0.003$) (Fig. 7).

4. Discussion

Previous research has highlighted the failure of various chemical insecticides to control *T. absoluta* populations, primarily due to their widespread and indiscriminate application (Roditakis et al, 2018; Guedes et al, 2019; Silva et al, 2019).

Compared to synthetic insecticides, EOs could be more eco-friendly due to low environmental persistence (Regnault-Roger et al, 2012), the lower toxicity to natural enemies and non-target organisms (Giunti et al, 2022), and the low probability of development of resistant insect populations due to their numerous active compounds (Isman, 2020). Despite their potential, the use of EOs is limited by their chemical and physical properties: indeed, EOs have poor water solubility and high volatility and are sensitive to environmental factors like light and temperature, which lead to instability and a loss of bioactivity (Rakmai, 2021; Devi et al, 2024). The use of nanoemulsions (NE) generally helps to mitigate these limitations and often enhances the biological activities of these compounds (Perinelli et al, 2022; Haghbayan et al, 2024; Khairan et al, 2024).

The primary objective of this study was to develop a garlic nano-emulsion characterized by a high concentration of active ingredients (15%) and a low surfactant percentage (5%), while maintaining long-term stability and a small particle size. This was achieved through the application of both high- and low-pressure microfluidization techniques. In contrast to many previous studies that produced nanoemulsions with lower EO content, this research successfully formulated two nanoemulsions with higher a.i. concentrations. The LP nanoemulsion was produced under a low pressure of 2,000 psi, while the HP one was achieved using a pressure of 20,000 psi.

The fabrication methods for nanoemulsions can be categorized into two main approaches: top-down and bottom-up. Top-down approaches focus on the disruption of the oil phase into uniformly sized, fine droplets, achieving homogeneity through mechanical or physical processes. In contrast, bottom-up approaches involve the direct assembly of molecular building blocks into structured systems,

guiding the formation of nanoemulsions from the molecular level (Donsi and Ferrari, 2016). In our study a combination of the two approaches was used. Indeed, a coarse emulsion obtained in the first step of fabrication methods we used, was obtained by self-emulsification process (bottom-up approach) and then it was subjected to different amounts of pressure (top-down approach). As reported by Modafferi et al., (2024) the use of hi-energy process to develop garlic EO-based nanoformulations allowed us to obtain particle sizes below 200 nm, remaining stable for up to three months.

The synthesized nanoemulsions were subsequently tested in the laboratory to assess: (i) the insecticidal effects on *T. absoluta* larvae, (ii) the possible side effects on the predator *N. tenuis*, through two different application methods (iii) the potential phytotoxicity on tomato plants and (iv) impact on the bacterial communities of *T. absoluta* larvae.

Our study highlighted the effective insecticidal activity of GEO against the tomato borer, *T. absoluta*. The results obtained with the LP nanoemulsion are similar to those of HP one regarding the insecticidal effect. Both exhibited acute toxicity against *T. absoluta* second instar larvae 72 h after treatment, with an LD₉₀ of 0.886 % for LP and 1.062 % for HP nano-formulation. We observed 100% mortality of larvae exposed to garlic treatments at the highest tested concentrations for both nanoemulsions. In detail, the LP nanoformulation determined 100% mortality at the three highest doses (i.e. 1%, 2% and 2.5%), while the HP one showed the complete mortality only at the 2.5% concentration.

The results we obtained are similar to those reported by Ricupero et al. (2022), where garlic EO caused 77.78% mortality of second instar of *T. absoluta* larvae at 72h after the treatment at a concentration of 0.124%, and 100% mortality at a concentration of 3%.

The use of garlic extracts, including also EO and main compounds, have been well investigated as insecticide by plant spraying, fumigation or topically applied (Denoirjean et al., 2022) against a series

of insect groups, including Hemiptera (Modafferi et al., 2024a), Lepidoptera (Ricupero et al., 2022; Ben Abdallah et al., 2023; Giuliano et al., 2024), termites (Srivastava et al., 2021), Coleoptera (Yang et al., 2010; Plata-Rueda et al., 2017), mosquitoes (Thomas and Callaghan, 1999), and eriophyid mites (Mossa et al., 2018). The insecticidal activity of garlic EO can be attributable to different mechanisms affecting insect physiology such as the digestive enzymes, the protein synthesis and the energy metabolism (Shahriari & Sahebzadeh, 2017; Wu et al., 2020).

Regarding *T. absoluta*, other EOs, including citrus (Campolo 2017), cardamom (Chegini and Abbasipour 2017) and ajwain (Piri 2020) EO demonstrated significant insecticidal efficacy against this pest.

Toxicity trials toward *N. tenuis* showed that the adult mortality rate at 72h was low for both nanoemulsions, regardless of the application method. In the ingestion trials, the HP nanoemulsion caused 12.5% mortality and the LP one 16% mortality, while in the topical application, the HP and the LP formulations caused 11.7 and 16.3% mortality, respectively. In contrast, acetamiprid was significantly more harmful to *N. tenuis*, resulting in high mortality rates (86% and 60%) of exposed adult mirids with the spray and the topical application, respectively. According to Ricupero et al. (2022), garlic essential oil-based nanoemulsion showed low levels of toxicity towards *N. tenuis*, despite the exposure affected the produced progeny. Furthermore, a study on *N. tenuis* carried out by Passos et al. (2022), obtained an LC₁₀ of 0.375 mg/mL and an LC₃₀ of 1.34 mg/mL for garlic EO topically applied. In addition, garlic EO negatively affected the female fecundity and impaired orientation of adult *N. tenuis*.

In phytotoxicity tests HP nanoemulsion did not show significant differences compared to the control after 7 days from application, since the LD₃₀ and LD₉₀ induced the 8.33% and 12.5% of leaves damage, respectively. On the other hand, the LP formulation caused significant greater damage on tomato plant than the control, with 25% damaged leaves for the LD₃₀ and 33% for the LD₉₀.

Our results differ from those reported by Ricupero et al. (2022), who observed no signs of toxicity on tomato plants sprayed with a garlic nanoemulsion. Due to their extremely heterogeneous set of secondary metabolites, EO can have different effects on plants, which also depend on the concentration and type of formulation used (Campolo et al, 2017).

Given the key role of microbiota on host fitness of insects, including *T. absoluta* (Chen et al., 2024; Lateef et al., 2024; Shang et al., 2024), the influence of the GEO nanoemulsions on the microbiota of pest larvae was evaluated. No significant differences in the abundance or diversity (Alpha diversity) of the associated bacteria were observed among treatments, either 24h or 72h after the treatment. However, the results revealed a shift in the bacterial community structure (Beta diversity), which was evident 72h after the treatments. Additionally, no significant differences were detected between the 24- and 72-hour time points within individual treatments, suggesting that the observed variations were not attributable to time.

The microbiota composition observed in larvae treated with water was consistent with findings from previous studies (e.g.: Lateef et al., 2024; Wang et al., 2022), albeit with some variations in prevalence. The bacterial community was predominantly dominated by bacteria of the family Enterobacteriaceae, Sphingomonadaceae, Moraxellaceae, Comamonadaceae, Pseudomonadaceae and Xanthomonadaceae. After 72h, two taxa, *Staphylococcus* and *Blastomonas*, were significantly more prevalent in EO-treated samples compared to untreated ones. Both taxa were predominantly found in treated samples, whereas they were minimally represented in untreated ones. Species of the genus *Staphylococcus* have been identified in recent studies characterizing the bacterial communities of *T. absoluta* eggs (Banadkuki et al., 2024; Bi et al 2023) and larvae (Chen et al., 2024; Shang et al., 2024). Notably, one of the species found in these studies, *Staphylococcus hominis* showed ability to tolerate the insecticide chlorantraniliprole (Chen et al., 2024). Another species, *S. gallinarum* was suggested to promote the synthesis of detoxification metabolic enzymes and degrade plant defense



compounds (Shang et al., 2024). A similar role in degrading toxic substances might be hypothesized for the *Staphylococcus* spp. identified in this study following EO treatment. The second taxon that significantly differed between treated and untreated samples, *Blastomonas*, has not previously been identified as a member of the bacterial communities associated with *T. absoluta*, to the best of our knowledge. It may represent an opportunistic bacterium that could have taken advantage of the decrease in certain bacteria observed 24h after treatment, even if not statistically significant, thereby creating a niche that allowed its proliferation.

Overall, the structural alterations observed in the microbiota of *T. absoluta* larvae are likely attributable to treatment with garlic EO, which is known for its antimicrobial properties. Garlic EO has been shown to be effective against a broad range of microorganisms, including gram-positive, gram-negative, and acid-fast bacteria (e.g. Ross et al., 2001; Behbahani & Fooladi, 2018). The sulfides present in garlic EO, particularly diallyl disulfide and diallyl trisulfide, have been identified as the primary compounds responsible for this antimicrobial activity (Casella et al., 2012). While gut dysbiosis following garlic treatments has been scarcely explored, similar microbiota alterations were observed in cockroaches exposed to garlic powder (Oladunni et al., 2023).

5. Conclusions

Despite observing phytotoxicity symptoms in tomato plants treated with the GEO nanoemulsion, the effectiveness of both formulations against *T. absoluta* and their low toxicity to the predator *N. tenuis* suggest that GEO nanoformulations could be a feasible option for managing *T. absoluta* populations in the field. However, the sub-lethal and disturbance effects on *N. tenuis* need further investigation in field conditions, where the predator's exposure to the substances is reduced. Additionally, more extensive research is needed to develop and test nanoformulations that can guarantee negligible levels of phytotoxicity for field crops.

6. References

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Table 1: Physical characterization of garlic essential oil nanoemulsions over time

Essential oil Type	Time	Average particle size (nm)	Polydispersity Index	Zeta (mV)
Garlic (2K PSI)	24 hours	163.5 ± 0.96	0.112 ± 0.02	-21.167 ± 0.75
	1 week	162.3 ± 1.08	0.096 ± 0.02	-25.133 ± 1.01
	1 month	173.6 ± 2.29	0.137 ± 0.02	-32.300 ± 0.46
	3 month	167.37 ± 0.65	0.182 ± 0.00	-31.4 ± 0.36
Garlic (20K PSI)	24 hours	109.77 ± 0.35	0.191 ± 0.00	-20.033 ± 1.12
	1 week	128.47 ± 0.70	0.200 ± 0.01	-24.233 ± 0.15
	1 month	152.07 ± 0.72	0.194 ± 0.006	-38.733 ± 0.68
	3 month	158.43 ± 1.55	0.192 ± 0.002	--

Table 2. Acute toxicity of low-pressure and high-pressure garlic EO nanoformulations against second-instar larvae of *T. absoluta* at 24, 48, and 72h after treatment. Values were considered significantly different if their 95% fiducial limits did not overlap.

Nanoformulations	Time	LD ₅₀	LD ₉₀	χ^2 (df)	<i>p</i>
LP		0.489 (0.401-0.579) _a	1.449 (1.136-2.121) _a	185.339 (208)	0.868
HP	24	0.496 (0.409-0.585) _a	1.419 (1.123-2.037) _a	197.141 (208)	0.695
LP		0.394 (0.309-0.473) _a	1.190 (0.940-1.736) _a	189.030 (208)	0.823
HP	48	0.420 (0.334-0.501) _a	1.263 (0.995-1.845) _a	187.206 (208)	0.847
LP		0.360 (0.289-0.423) _a	0.886 (0.725-1.227) _a	148.185 (208)	0.999
HP	72	0.359 (0.276-0.432) _a	1.062 (0.845-1.535) _a	196.461 (208)	0.707

7. Figure captions

Figure 1. Effect of different application rates of LP garlic nanoemulsions on second-instar *Tuta absoluta* larval mortality at 24, 48, and 72 hours (2,000 PSI pressure)

Figure 2. Effect of different application rates of HP garlic nanoemulsions on second-instar *Tuta absoluta* larval mortality at 24, 48, and 72 hours (20,000 PSI pressure).

Figure 3. *Nesidiocoris tenuis* mortality after spray application of HP and LP nanoemulsions, compared with acetamiprid. Values were considered significantly different if their 95% fiducial limits did not overlap.

Figure 4. *Nesidiocoris tenuis* mortality after topical application of HP and LP nanoemulsions, compared with acetamiprid. Values were considered significantly different if their 95% fiducial limits did not overlap.

Figure 5. Percentage of damage in tomato plants treated with LP nano-formulation. Symptoms appeared 24 h after treatment, with damage increasing over 7 days.

Figure 6. Percentage of damage in tomato plants treated with HP nano-formulation. Symptoms appeared 48 h after treatment, with damage increasing over 7 days.

Figure 7. Principle coordinate analysis (PCoA) of the bacterial communities in treated and untreated *Tuta absoluta* after 24h and 72h, based on the Bray–Curtis dissimilarity.

Fig. 1

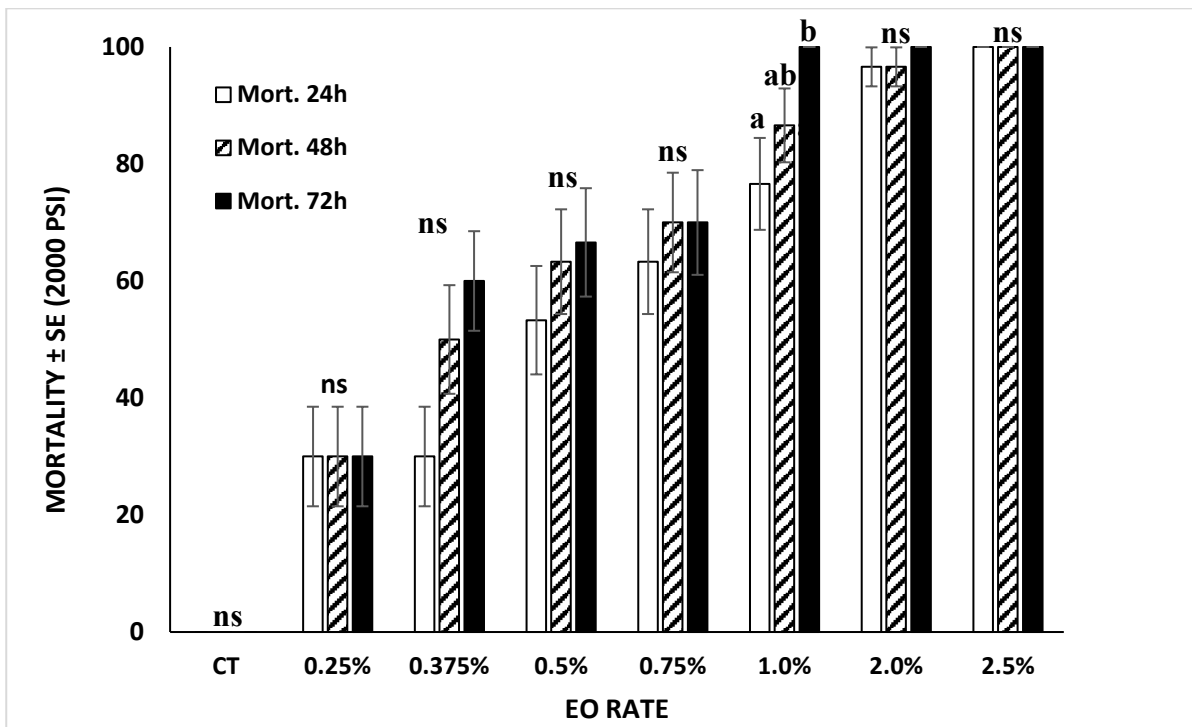


Fig. 2

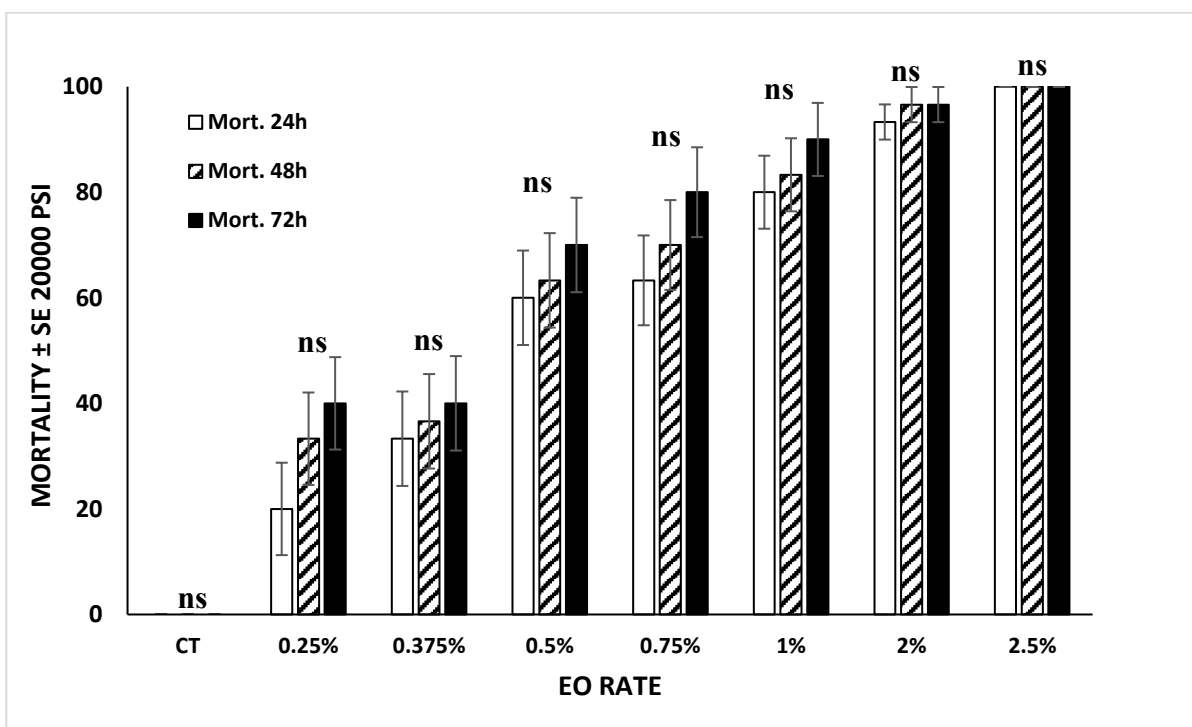


Fig. 3

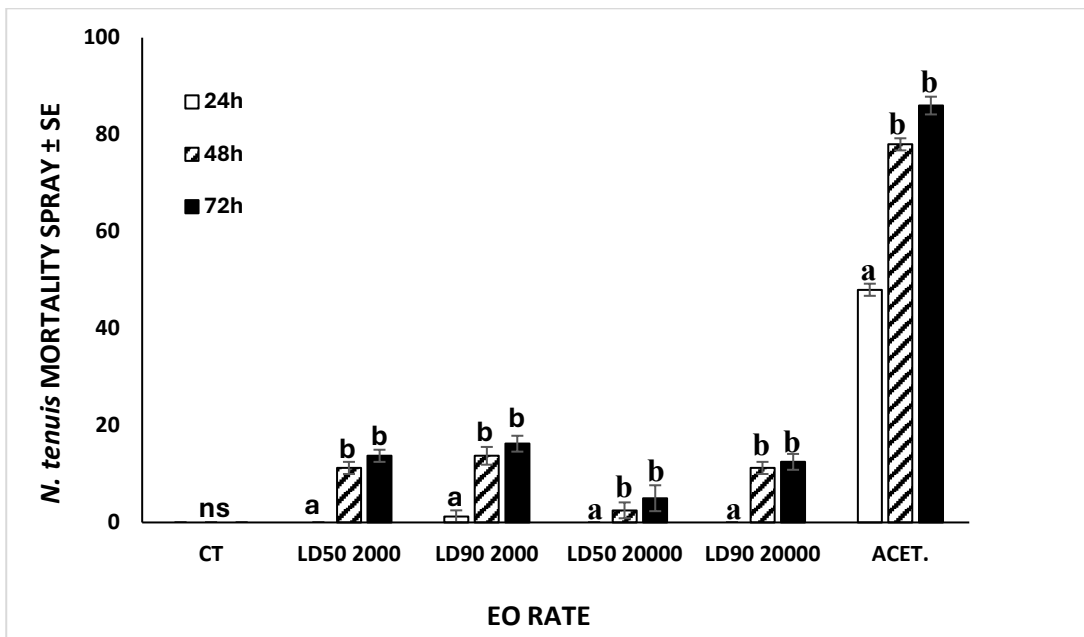


Fig. 4

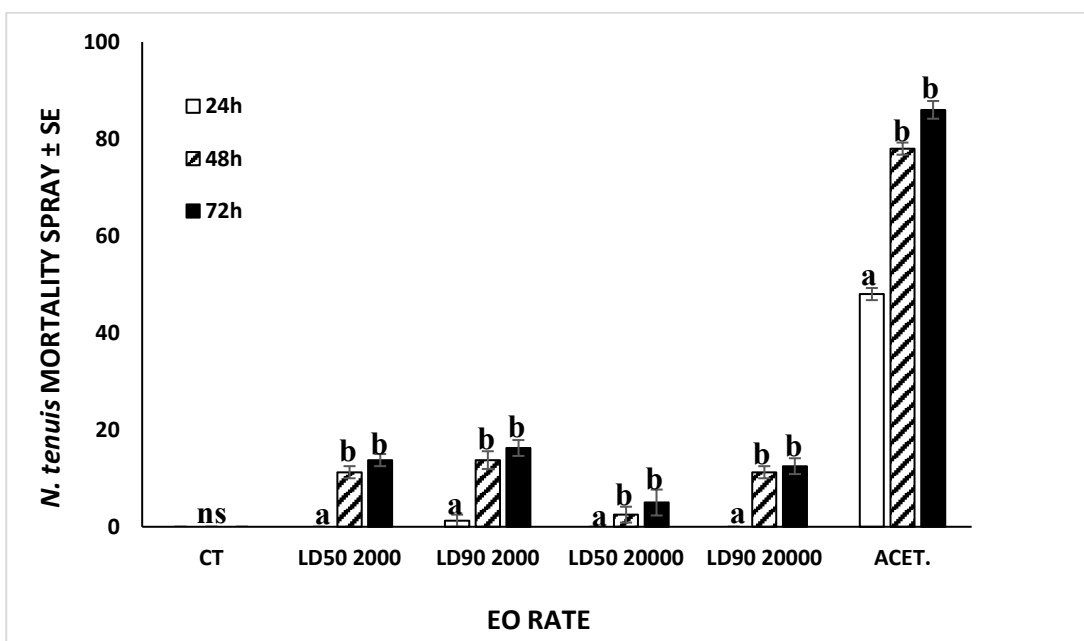


Fig. 5

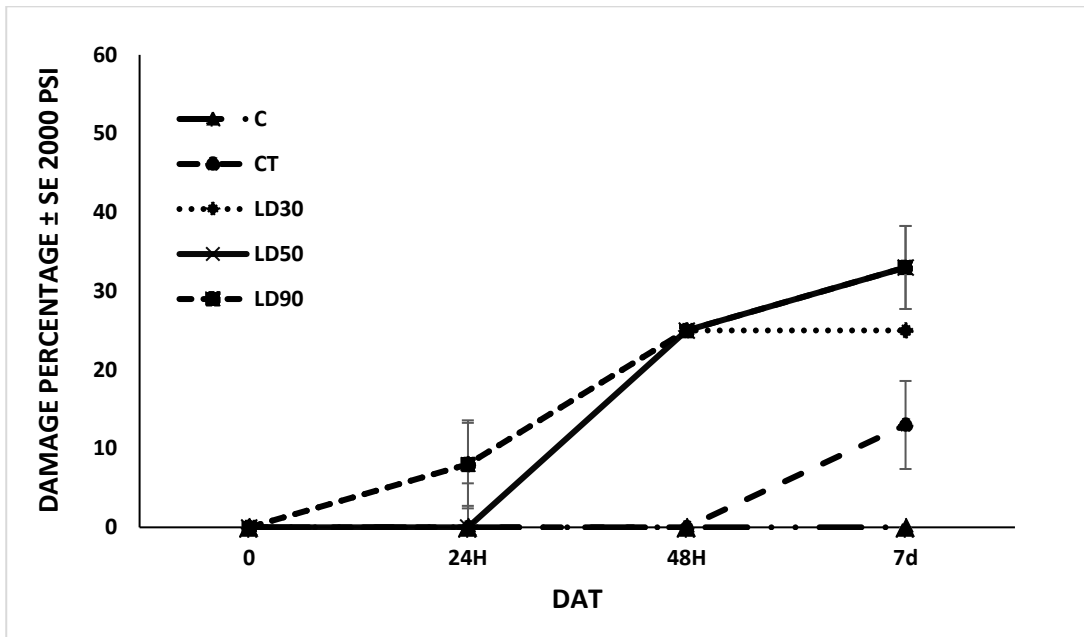


Fig. 6

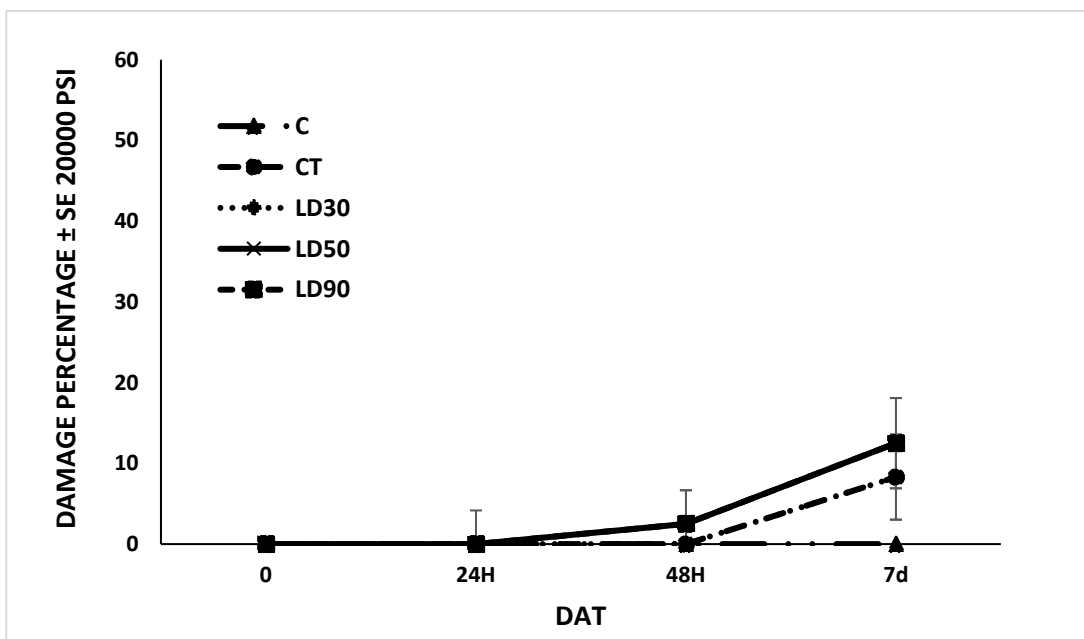
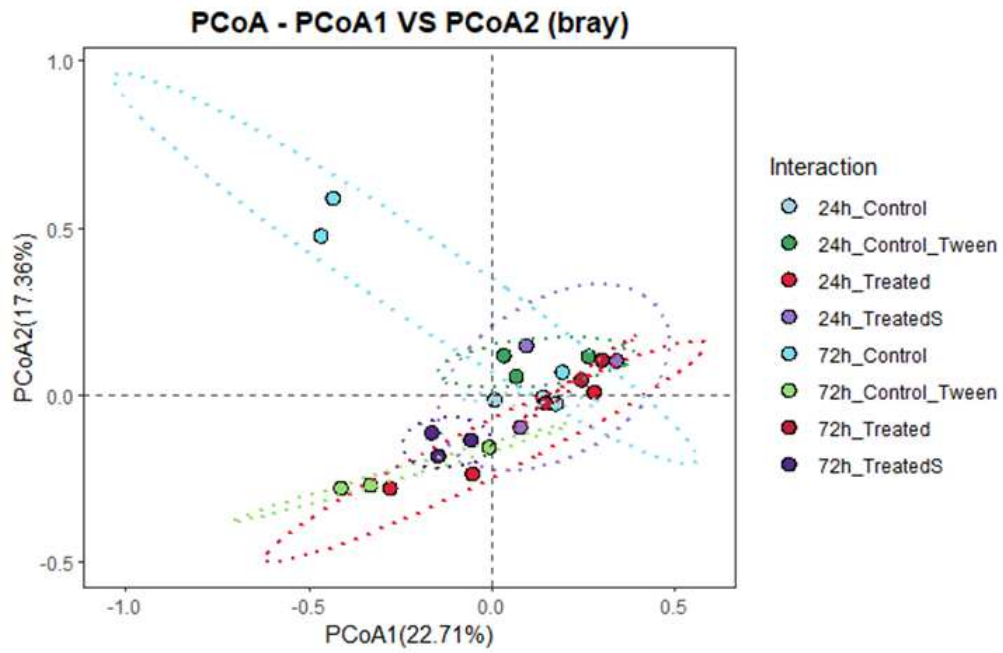


Fig. 7





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In submissions to Crop Protection

V. **Garlic essential oil nanoemulsion for sustainable management of *Bemisia tabaci* and phytotoxicity evaluation in four key horticultural crops**

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HIGHLIGHTS

- Garlic EO nanoemulsions effectively control *Bemisia tabaci* nymphs and adults
- High-pressure microfluidization ensures uniform particle size and good stability
- Garlic EO nanoemulsion significantly reduce oviposition by exposed *B. tabaci* adults
- Phytotoxic effects are crop-dependent, with minimal damage at LC50 concentrations
- This study supports eco-friendly pest control solutions for horticultural crops

Abstract

Bemisia tabaci is a pest affecting many crops globally, leading to significant agricultural losses through direct feeding damage, honeydew deposition, and virus transmission. The overuse of synthetic insecticides has led to resistant populations, necessitating alternative control strategies. This study evaluates the insecticidal efficacy of a garlic (*Allium sativum*) essential oil (EO) nanoemulsion against different life stages of *B. tabaci* and its phytotoxic effects on cucumber, eggplant, tomato, and pepper. The nanoemulsion, developed using high-pressure microfluidization, demonstrated high particle uniformity ($PDI < 0.1$) and an average droplet size of 61.83 nm.

The garlic EO nanoemulsion was most effective against nymphs and adults, achieving over 80% and 90% mortality, respectively, at the highest concentrations tested. Furthermore, treated adults exhibited a marked decrease in egg laying. At the highest concentration, oviposition was almost completely inhibited. While ovicidal activity was limited, with less than 50% egg mortality observed at the maximum concentration, the combined impact on adult mortality and reduced offspring highlighted the nanoemulsion's potential to disrupt the pest's population dynamics. No significant phytotoxicity at LC50 across all tested crops was revealed, while LC90 concentrations caused moderate damage to cucumber and eggplant but were less detrimental to tomato and pepper.

These findings highlight the potential of garlic EO nanoemulsions as an eco-friendly alternative to conventional insecticides. by targeting multiple life stages and reducing reproductive success, garlic EO nanoemulsions can play a vital role in integrated pest management strategies, addressing key challenges such as resistance and environmental impact.



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Key words: ecofriendly insecticide; High-pressure homogenization, biological control, botanicals

1. Introduction

Bemisia tabaci Gennadius (Hemiptera: Aleyrodidae), known as the silverleaf whitefly or the sweet potato whitefly, is one of the most important and widespread pests in open field and greenhouse cropping systems (De Barro et al., 2011; Fan and Petitt, 1998; Oliveira et al., 2001). This pest is highly polyphagous and feeds on over 600 plants, including ornamental and horticultural plants (De Barro et al., 2011; Oliveira et al., 2001; Simmons et al., 2008). Damage includes direct feeding damage through sap removal, extensive honeydew deposition that promotes the growth of sooty mold, and the transmission of several plant viruses (De Barro et al., 2011; Jones, 2003). Among these, the transmission of the geminivirus Tomato Yellow Leaf Curl Virus (TYLCV) in tomatoes is particularly devastating. The control of *B. tabaci* populations and vectored plant viruses is challenging due to the diversity of genetic clades, their rapid life cycle with overlapping generations, their habit of residing mainly on the underside of leaves, and their ability to develop resistance to synthetic insecticides (De Barro et al., 2011; Denholm et al., 2008; Horowitz et al., 2020). Despite advancements in pest management, such as the paradigmatic example of using biological control in greenhouse crops in southeastern Spain (van Lenteren et al., 2018), conventional insecticides remain the primary method of control for *B. tabaci*, particularly in horticultural production systems across various regions of the world (Mahmood et al., 2023). However, their over use have triggered resistance in some *B. tabaci* populations (Basit, 2019; Horowitz et al., 2020; Liang et al., 2012). Resistance for modulators of nicotinic acetylcholine receptors (e.g., neonicotinoids), molecules that act on insect nerves and muscles (e.g., pyrethroids, organophosphates and diamides), and a wide variety of growth-regulating insecticides (D'angelo et al., 2018; Roditakis et al., 2009) have been already documented. Furthermore, the increasingly frequent demand for pesticide-free food by consumers, the withdrawal of several active substances

from the market, and the negative effects of synthetic insecticides on the environment and non-target insects (Ahmed et al., 2024; Biondi et al., 2013, 2012) has encouraged researchers to search for alternative solutions for the control of crop pests (Giuliano et al., 2024; Giunti et al., 2019). If appropriately formulated and used, botanical insecticides can offer a sustainable and ecological approach to pest management, aligning with integrated pest management (IPM) principles and organic farming practices (Campolo et al., 2020; Pavela and Benelli, 2016). In particular, interest in plant extracts such as essential oils (EOs) is growing year after year, as demonstrated by the increasing number of research on EO-based insecticides tested against several insect pests and vectors, including Hemiptera (Modafferi et al., 2024), Lepidoptera (Ben Abdallah et al., 2023; Giuliano et al., 2024; Ricupero et al., 2022), Termites (Srivastava et al., 2021), Coleoptera (Pérez et al., 2010; Plata-Rueda et al., 2017), Mosquitoes (Thomas and Callaghan, 1999) and Eriophyid (Mossa et al., 2018). Indeed, EOs have a broad-spectrum activity due to their complex chemical compositions and the presence of various bioactive compounds, reducing the possible onset of insect resistance. Thus, their insecticidal activity, coupled with the reduced risk of insect resistance development, make EOs promising tools for controlling crop pests. On the other hand, the usual problems associated with EOs in field conditions, such as low stability, degradability, poor persistence, and phytotoxicity on plants, decreased their marketability. However, proper formulation can solve or reduce those criticisms (Campolo et al., 2020, 2017; Giunti et al., 2019). On this basis, this study aimed to evaluate the insecticidal activity of garlic (*Allium sativum* L.) EO based nano-emulsion was obtained using the high-pressure micro-fluidization technique to include a high amount of a.i. (i.e., 15%) and low surfactant content against eggs, nymphs, and adults of *B. tabaci*. The phytotoxicity of the nano-formulation was also evaluated on four major horticultural crops of global importance: cucumber, eggplant, tomato, and pepper.

2. Materials and method

2.1. Insects and plants rearing

The laboratory clade of *B. tabaci* MED (Mediterranean group) used in this study was mass-reared in the agricultural research center Agrigeos s.r.l. (Acireale, Italy). In insect-rearing cages (BugDorm®), eggplant and bell pepper plants were used as hosts. Adult specimens were collected using a mechanical aspirator and placed in ventilated plastic tubes (Falcon®, 50 mL) before being used in the experiments. Bell pepper plants (*Capsicum annuum* L. cv Makko F1), eggplants (*Solanum melongena* L. cv Giada F1), tomato plants (*Solanum lycopersicum* L. cv Marmande), and cucumber plants (*Cucurbita pepo* L. cv Diamant F1) were used in the experiments. All plants were grown from untreated seeds and planted in pots filled with organic soil. Both insects and plants used in the experiments were maintained under controlled conditions, including a temperature of $24 \pm 2^\circ\text{C}$, relative humidity (RH) of $50 \pm 10\%$, and a photoperiod of 14:10 h (L:D). All experiments were conducted under controlled environmental conditions in growth chambers ($25 \pm 2^\circ\text{C}$, $60 \pm 10\%$ RH, 14:10 h L:D) and were repeated twice on different days. Four replicates for every tested concentration of garlic EO nanoemulsion, as well as for controls, were provided.

2.2. Development and characterization of Garlic EO-based nano-formulation

The *A. sativum* EO used in this study originated from the same EO batch employed by Modafferi et al. (2024), which reported a detailed list of identified compounds. The EO primarily consisted of sulfur compounds, with diallyl disulfide (37.26%), diallyl trisulfide (28.15%), diallyl tetrasulfide (12.20%), 1-allyl-3-(2-(allylthio)propyl)trisulfane (6.69%), and diallyl sulfide (5.84%) as the most abundant detected compounds. The garlic nano-emulsion was obtained using high-energy emulsification process as described by Modafferi et al. (2024) with some modifications. In detail, in

the first step, a raw emulsion was obtained through the self-emulsification method by mixing (30 min at 7,000 rpm at 25 ± 2 °C) EO and Tween 80® [polyoxyethylene (20) sorbitan monooleate, (Sigma-Aldrich, Munich, Germany)] 7 (ratio 3:1 w:w). After that, double-distilled water was slowly added (1 mL min⁻¹) to the oil mixture (ratio 4:1 w:w). The mixture (EO 15% w/w; Tween 80® 5% w/w; water 80% w/w) was then stirred for 3h at 7,000 RPM. Finally, aliquots of the obtained raw emulsion were homogenized by a high pressure microfluidizer (LM20 Microfluidizer™ Processor, USA) for 3 cycles at 30,000 (HP formulation). The interaction chamber was immersed in an ice bath to avoid heat degradation of the EOs during the high-pressure homogenization. A Dynamic Light Scattering (DLS) apparatus (Zetasizer Nano© - Malvern Instruments) was employed to analyze the physical properties of the nano-emulsion, precisely measuring droplet size and polydispersity index (PDI). The samples were diluted with double-distilled water for DLS measurements at 1:400 (v:v). Seven different concentrations of garlic EO (0.25, 0.5, 0.75, 1, 1.5, 2, and 2.5%) were tested, and water was used as negative control. The same concentrations were tested on whitefly eggs, nymphs, and adults (see following sections). Based on preliminary observations, the tested concentrations were chosen to identify the dosage, resulting in mortality levels ranging between 10% and 90% of the exposed whitefly stages. This mortality range was selected to accomplish the Probit analysis requirement to adequately calculate the concentration of EO required to kill 50% (LC50) and 90% (LC90) of the exposed egg, nymph, and adult whiteflies. The Henderson-Tilton (1955) formula was used to uniform the population between the different replications and doses.

2.3. Ovicidal bioassay

Bell pepper plants were singly placed in a sterile plastic cylinder arena (10 x 25 cm). Afterward, 20 *B. tabaci* adults were collected and released into each arena using a mouth aspirator (Omnes artes,

Italy). Each cylinder containing the plant and the adult pests was then closed with a perforated lid made of fine mesh fabric to prevent insect escape and allow ventilation. After 2 days, the *B. tabaci* adults were removed, and eggs were laid and counted using a binocular microscope. Thus, the pepper plants containing the eggs were dipped in the diluted nano-formulation at different concentrations for 10 seconds and left to air dry for 45 minutes. Four replicates were conducted for every tested concentration: negative control (water) and positive control (Acetamiprid at 0.5 L/ha; Kestrel®, Nufarm Italia s.r.l., Italy). Mortality was recorded daily for seven days following the treatment, counting the number of dead eggs per leaf and the number of hatched 1st instar nymphs (N1). The eggs were considered dead when they appeared dry.

2.4. Toxicity against *Bemisia tabaci* nymphs

In this experiment, the methodology used to obtain nymphs was the same as described in the experiment with eggs. For each tested concentration, including negative control (water) and positive control (Acetamiprid at 0.5 L/ha; Kestrel®, Nufarm Italia s.r.l., Italy), four replicates (i.e., plants) were conducted. Two days after being released, the *B. tabaci* adults were removed, and after 7 days, the number of 2nd instars (N2) was counted using a binocular microscope. Thereafter, the pepper plants containing the 2nd instars were dipped in the different concentrations of nano-emulsion for 10 seconds and left to air dry for 45 minutes. Mortality was recorded after three days, counting the number of dead specimens. The nymphs were considered dead when they appeared dry.

2.5. Toxicity against *Bemisia tabaci* adults

In this experiment, the methodology recommended by IRAC was applied (IRAC, 2009). Bell pepper leaf discs were cut and dipped for 10 seconds in the garlic nano-formulation at different concentrations and then left to air dry for 45 minutes. The dry leaf discs were then placed in small Petri dishes (diameter 60 mm) upon a layer of water-agar to keep the leaf in good moisture condition for a few days. Ten *B. tabaci* adults (sex-ratio: 1:1), previously aspirated using a mechanical aspirator, were released into each Petri dish. Each Petri dish was closed with a perforated lid and a fine mesh net to avoid the fumigant effect and insect escape. Four replicates (40 adults) were provided for each concentration tested, including negative (water) and positive (Kestrel: acetamiprid at highest label dose) controls. Mortality was recorded after 3 days from the treatment, counting the number of dead adults and the number of laid eggs. The adults were considered dead when they did not move if stimulated with a fine brush.

2.6. Phytotoxic effects of garlic nano-formulations on four different plants

A specific trial was set up to evaluate the non-target effects of the garlic EO-based nano-formulation on plants. In this study, the LC50 and LC90 estimated for *B. tabaci* nymphs were tested. Four different *B. tabaci* host plants were selected: tomato (*Solanum lycopersicum* L.), eggplant (*Solanum melongena* L.), zucchini (*Cucurbita pepo* L.), and bell pepper (*Capsicum annum* L.). These plants were sprayed with the garlic nano-emulsion until runoff. They were kept under controlled conditions in growth chambers (25± 2 °C, 60± 10% RH, 14:10 L:D) until the end of the experiment (21 days after treatment). Each tested concentration, including the negative control (water), was replicated four times (i.e., 4 plants for concentration). The effects on plants of the developed formulations were

estimated by using the phytotoxicity index (P_i) according to Campolo et al. (2017) and calculated as follows:

$$P_i = \sum_{j=0}^n \left(\frac{DL_j}{TL} \times \frac{DC}{n-1} \right)$$

where DL is the number of damaged leaves for each damage severity class j , TL is the total number of leaves sprayed, DC is the damage severity class, and n is the number of damage severity classes (0 = no phytotoxicity damage; 1 = 25% of damaged leaf surface; 2 = 50% damaged leaf surface; 3 = 75% damaged leaf surface; 4 = 100% of damaged leaf surface). The calculated P_i ranges from 0 (no damage) to 1 (dead leaves). The damage on leaves was observed 3, 7, 14, and 21 days after treatment.

2.7. Data analysis

Mortality of different *B. tabaci* stages were subjected to ANOVA, and differences among means were compared with Tukey's Honestly Significant Difference (HSD) test at $P < 0.05$. Outliers have been identified using Cook's distance and studentized residuals. A second ANOVA model was used to evaluate the impact of the outliers on the analysis without including them. The models were compared using Akaike's Information Criterion (AIC) and Bayesian Information Criterion (BIC) to assess model parsimony and predictive performance. As both AIC and BIC consistently indicated that the model excluding outliers was more parsimonious, these data points were eliminated from the analyses. Normality and homogeneity of the variances were checked using the Shapiro–Wilk and Levene tests, respectively, before ANOVA. Since the ANOVA assumptions were met, it was unnecessary to transform the data. Mortality in EO-treated insects was corrected using the Henderson–Tilton formula.

A generalized linear model (GLM) was used to analyze the interactions between different dosages, days, and plant species in phytotoxicity bioassays. Tukey's HSD was used for multiple comparisons. All the data satisfied the GLM assumptions in terms of normality and homoscedasticity of variance ($p > 0.05$).

Probit analysis (Finney, 1947) was used to estimate the LD50 and LD90; values were considered significantly different if their 95% fiducial limits did not overlap. Statistics were carried out using IBM SPSS 19 for Probit analysis and R v4.3.2 (R Core Team, 2023) for all other analyses.

3. Results

3.1. Development and characterization of Garlic EO-based nano-formulation

The developed formulation exhibited an average particle size of 61.83 ± 0.43 nm, denoting the nanoscale dimension of the micelles. The low polydispersity index (PDI) of 0.09 ± 0.01 suggested a high uniformity among the distribution of nanoparticles (Fig. 1). These characteristics are critical for ensuring consistent performance, as uniform particle size can enhance bioavailability and stability. Moreover, no evidence of phase separation was observed throughout the experimental period, confirming the physical stability of the nano-emulsion system. This stability can be attributed to the optimized formulation process and potential stabilizing interactions within the system.

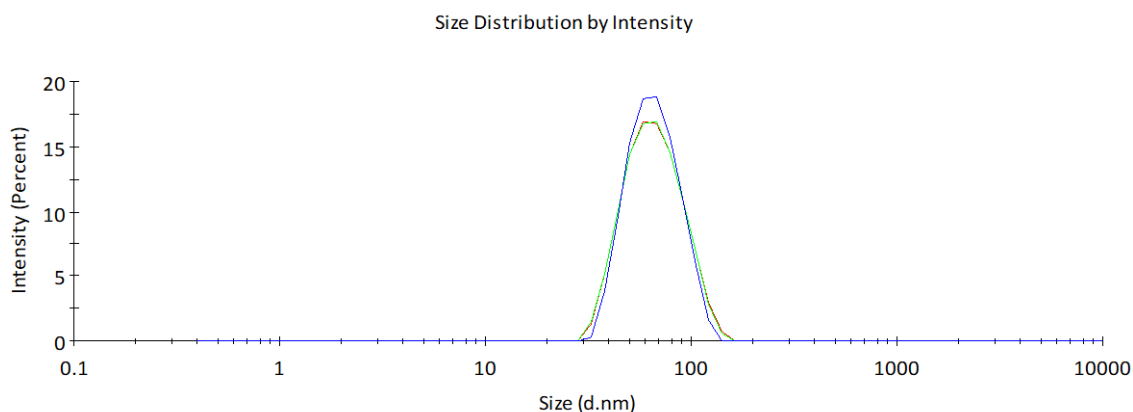


Figure 1: Size distribution of garlic EO-based nano-formulation droplets. Each color corresponds to a different replicate.

3.2. Insecticidal activity on *B. tabaci* eggs

The tested formulations did not exhibit strong ovicidal activity, with an average mortality rate reaching $50 \pm 4.6\%$ at the highest concentration (i.e., 2.5%). Indeed, only the lowest concentration showed statistically significant differences compared to the other tested concentrations, with a mortality rate lower than 10% ($F= 4.95$, $df= 1, 6$, $P < 0.05$)(Fig. 2). The $50.0 \pm 2.3\%$ of the eggs treated with acetamiprid did not hatch.

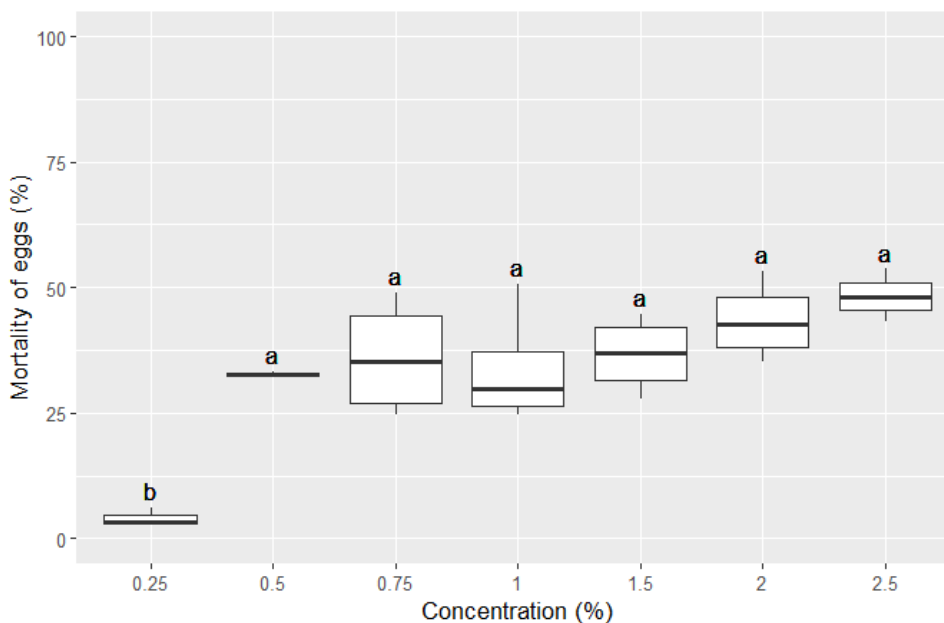


Figure 2: Box plots of the percentage mortality of *B. tabaci* eggs for the different concentrations of garlic EO nano-emulsion. For each concentration, four replicates were conducted. Letters denote significant differences among treatments according to Tukey multiple pairwise comparisons ($P < 0.05$).

3.3. Insecticidal activity on *B. tabaci* nymphs

Garlic EO-based nano-formulation showed high toxicity against second-instar nymphs of *B. tabaci*. During the 7-day observation period, the untreated control (water) exhibited no mortality, while in the positive control (acetamiprid), $88.1 \pm \%1.7$ of nymphs died. Through the Probit analysis calculation, we obtained an LD50 of 0.842 % (0.769-0.918) and an LD90 of 6.057 % (4.880-7.942) ($X^2= 12.573$; $df= 26$; $P= 0.986$) of the tested garlic EO-cased formulation. The mortality percentage significantly differed among the application rates used ($F= 28.15$; $df=1, 6$; $P< 0.01$). Three distinct groups were formed from the tested concentrations: the lowest concentration resulted in the lowest mortality rate; the three intermediate concentrations had intermediate mortality rates with no significant differences; and the three highest concentrations exhibited high comparable mortality rates, all above 70% and with no significant differences among them (Fig. 3).

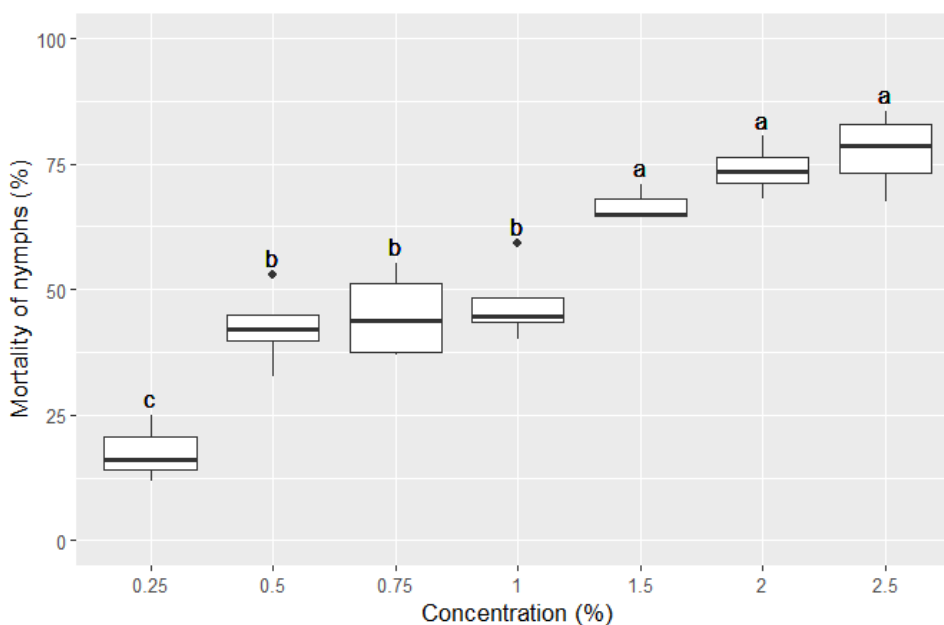


Figure 3. Box plots of the percentage mortality of *B. tabaci* nymphs for the different concentrations of garlic EO nano-formulation. For each concentration, four replicates were conducted. Letters

denoted significant differences among treatments according to Tukey multiple pairwise comparisons ($P < 0.05$).

3.4. Insecticidal activity on *B. tabaci* adults

Insecticidal activity on *B. tabaci* adults Garlic EO-based nano-formulation exhibited high toxicity against *B. tabaci* adults and the number of laid eggs. During the entire observation (3 days), no mortality was registered in the untreated control (water); instead, in the treated leaves with Acetamiprid and those treated with the highest dose, we recorded a mortality rate of 100% of adults. Furthermore, in the leaves treated with the second and third highest EO concentrations, $92 \pm 9,6\%$ and $90 \pm 8,2\%$ adult mortality, respectively, was achieved ($F = 36.57$, $df = 1, 8$, $P < 0.05$) (Fig. 4). The amounts of EO required to kill 50 and 90% of the exposed adults were 0.542 and 1.719 g/L respectively ($\chi^2 = 13.456$; $df = 26$; $P = 0.979$). Furthermore, in the leaves treated with the highest concentration of EO (i.e., 2.5%) and Acetamiprid, no eggs were counted; instead, in 2% and 1.5% of EO treatments, $0.39 \pm 0.2\%$ and $4.9 \pm 0.59\%$ of total laid eggs, respectively were counted ($F = 10.69$, $df = 1, 8$, $P < 0.05$) (Fig. 5).

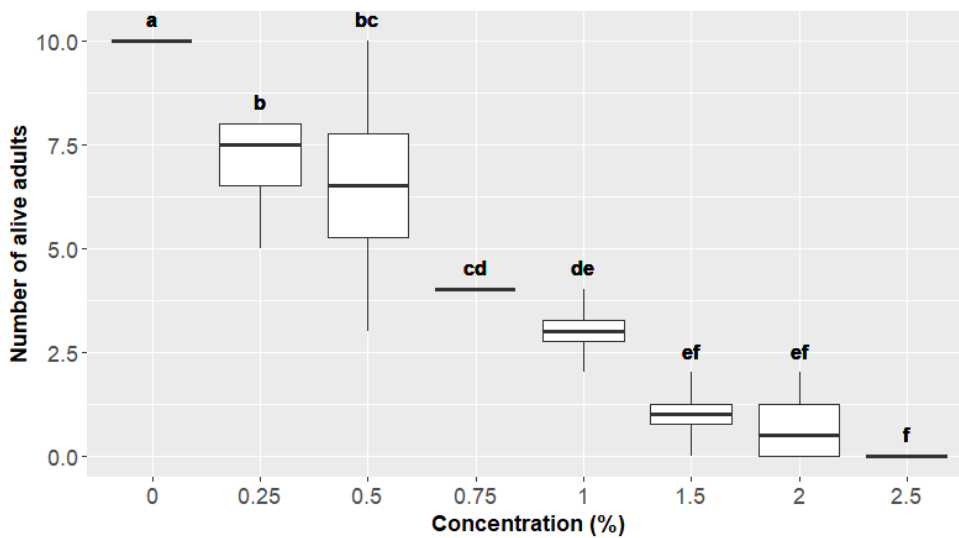


Figure 4. Box plots of the number of alive *B. tabaci* adults for the different concentrations of garlic EO nano-formulation, negative (i.e., 0) and positive control (i.e., acetamiprid). For each concentration, four replicates were conducted, each with ten adults. Letters denoted significant differences among treatments according to Tukey multiple pairwise comparisons ($P < 0.05$).

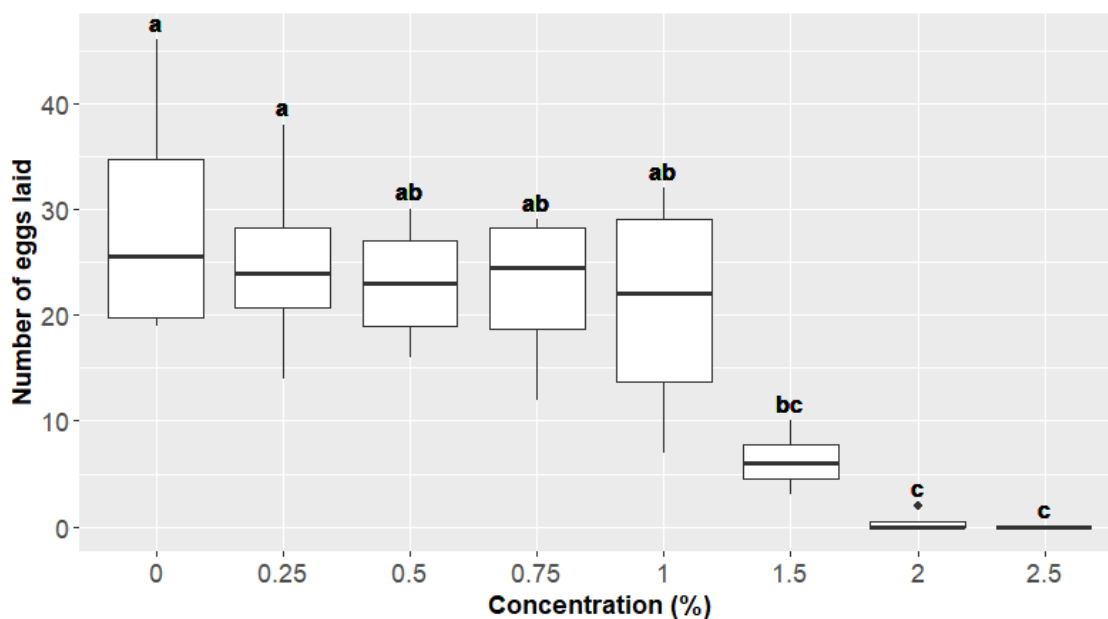


Figure 5. Box plots of the number of eggs laid by *B. tabaci* at the different concentrations of garlic EO nano-formulation, negative (i.e., 279 0) and positive control (acetamiprid). For each

concentration, four replicates were conducted. Letters denoted differences among 280 groups according to Tukey multiple pairwise comparisons ($P < 0.05$).

3.5. Phytotoxic effects

Symptoms related to phytotoxicity began to appear three days after treatment, with variable levels of damage depending on the crop plant. Figure 6 displays the results, while Table A.1 (Supplementary Table A1) provides a detailed account of the findings. Throughout the survey period, none of the control plants exhibited any damage. Phytotoxicity values remained below 0.7, reaching a peak of 0.67 in cucumber plants 7 days post-treatment with LC90. Regarding the plants treated with the LC50, no differences were detected between the four plants and the control across all four measurement days (3, 7, 14, and 21 days). Additionally, no differences were observed among the plants, although the cucumber exhibited higher damage levels than the others (Supplementary Table A1). The LC90 dose treatment resulted in highly variable damage levels among the plants. Cucumber showed the highest degree of damage overall, whereas pepper exhibited the least. The damage level in cucumber was consistently higher than the control across all four days of observation. In cucumber, eggplant, and tomato treated with LC90, phytotoxicity values decreased over time due to the formation of new leaves (Supplementary Table A1).

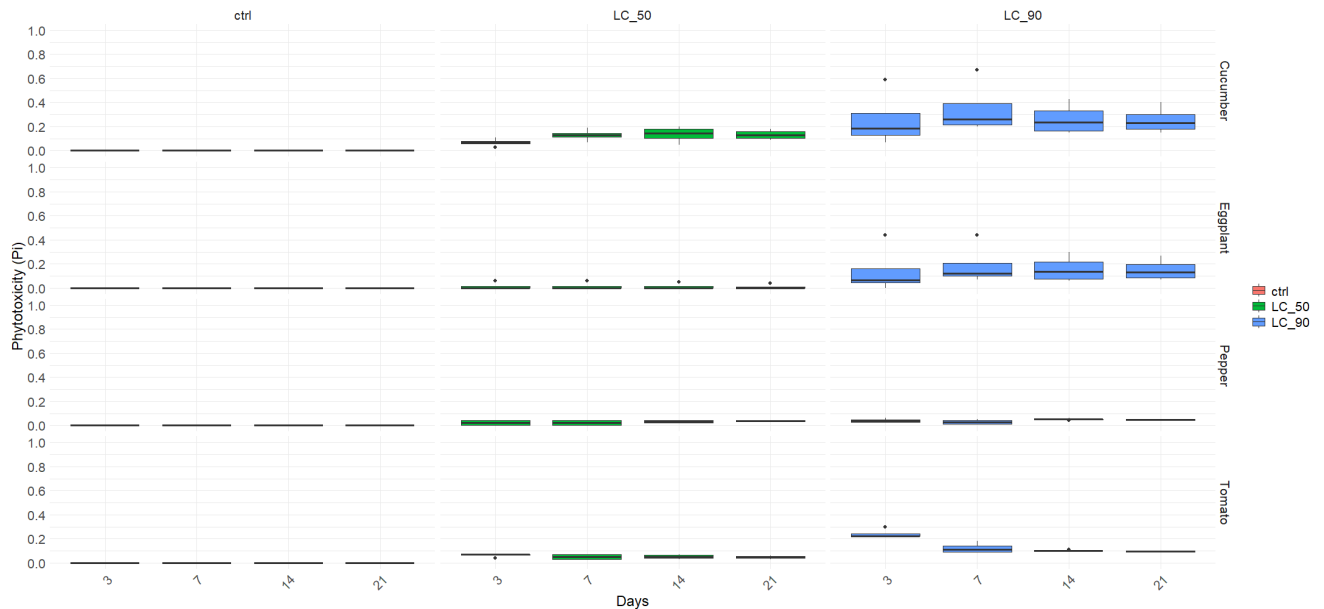


Figure 6: Boxplot of phytotoxicity values (P_i) over time. The x-axis shows the days post-treatment, and the boxplot colors indicate the concentrations tested. The graph is categorized into three groups for each tested plant: *ctrl* for negative control, *LC_50*, and *LC_90* for the tested concentration of garlic EO nano-formulation.

4. Discussion and conclusion

Our results demonstrate the potential of garlic essential oil nanoemulsions for use in horticultural crops, both for their effectiveness against the key pest *B. tabaci* and their low phytotoxicity in four major horticultural crops. These innovative findings provide valuable insights into the ongoing debate surrounding using essential oils in horticulture. Despite their potential as promising alternatives to conventional insecticides, several challenges hinder their widespread adoption for managing *B. tabaci* and other pests. One major issue is the variability in their composition, influenced by factors such as plant source, environmental conditions, and extraction methods, which can result in inconsistent efficacy. Additionally, essential oils' volatility and rapid degradation under field conditions limit their

long-term effectiveness. However, some of these drawbacks can be mitigated through nanotechnology, such as including EO in nano-delivery systems (Giunti et al., 2023). Therefore, garlic EO nanoemulsions emerge as a promising natural insecticide, primarily due to their well-documented insecticidal activity, which is attributed mainly to the presence of sulfur-containing compounds such as diallyl disulfide and diallyl trisulfide (Demeter et al., 2021). This EO acts through multiple mechanisms of action, including disruption of the insect nervous system by interfering with neurotransmitter function and ion channel activity, ultimately causing paralysis and death (Plata Rueda et al., 2017). In addition, it is not only an effective insecticide but also a potent repellent against pests, such as the rice weevil, *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) (Kanimozhi et al., 2023). The nano-emulsion developed in this study exhibited a remarkably small particle size (61.83 nm) with a high particle uniformity (PDI <0.01).

High-pressure microfluidization as a key processing technique further enhanced the nano-emulsion's characteristics. Increasing homogenization pressure intensifies the shear forces acting on the system, resulting in finer droplet sizes. Similarly, increasing the number of nano-emulsion cycles through the microchannels enhances energy input and improves uniformity, leading to smaller droplets (Jasmina et al., 2017). For instance, Ricupero et al. (2022) successfully developed a garlic EO nano-emulsion using a self-emulsifying process combined with sonication. However, their formulation displayed larger droplet sizes (176.23 ± 0.9 nm) and lower uniformity, as indicated by a polydispersity index (PDI) 0.18. In contrast, the nano-insecticides formulated by Modafferi et al. (2024), using the same ingredients but with five microfluidization cycles instead of three, achieved droplet sizes and homogeneity comparable to those of this study. These nano-insecticides, leveraging the advantages of nano-emulsions, offer improved bioavailability, targeted action, and reduced environmental impact, addressing critical challenges in pest management.

Our results highlighted that the developed nano-formulation effectively controlled *B. tabaci*, although to different degrees, depending on the tested life stages. Garlic EO nano-emulsions can effectively control the adult population of *B. tabaci*, with three out of seven concentrations causing more than 80% mortality. These concentrations (i.e., 1.5, 2, and 2.5%) also resulted in high nymph mortality rates. However, the same concentrations were less effective against eggs. Despite the common perception of insect eggs as a very vulnerable life stage, their response to insecticides has rarely been the subject of scientific investigation. Furthermore, the immobile nature and tendency to be hidden in protected areas make it challenging for eggs to target with insecticide treatments. Additionally, the structure of the eggs can shield the developing embryos and hinder insecticide penetration (Beament, 1952; Koppel et al., 2011). Our findings align with those of Hussein (2017), who tested different raw emulsions, produced with three EOs (garlic, thyme and cumi) and TritonX as a surfactant, against various *B. tabaci* instars. In that study, garlic EO reduced egg hatchability by approximately 50%, while nymphal mortality reached 66.43% at 1,000 ppm. Hussein (2017) also noted that immature stages were more sensitive to the tested E than eggs and pupae. Similar results were obtained by da Silva Santana et al. (2022) by treating *B. tabaci* eggs with an acetone- *Mansoa alliacea* EO solution whose main compounds are diallyl trisulfide (52.8%) and diallyl disulfide (33.9%). The spray application of LC90 and LC50 estimated for nymphs reduced the hatchability by approximately 50% while the same concentration resulted in both adult and oviposition deterrence. Similar results were obtained by treating *B. tabaci* eggs with 0.125-0.5% of *Thymus vulgaris*, *Pogostemon cablin*, and *Corymbia citriodora* EOs formulated with 0.5% of Tween 20 (Yang et al., 2010). The low susceptibility of eggs was highlighted also in other pests. As an example, eggs of *Phthorimaea absoluta* Meyrick (Lepidoptera: Gelechiidae) treated with nanoparticles loaded with citrus peel EOs exhibited a lower toxicity against this instar rather than to larvae (Campolo et al., 2017). Comparable

results were observed with garlic EO nano-emulsion, where the concentration needed to achieve over 80% larval mortality was consistently less effective against eggs (Ricupero et al., 2022).

Here, applying the developed nano-formulation against *B. tabaci* 2nd instar nymphs and adults resulted in dose-dependent mortality, achieving maximum efficacy of over 80% and 90%, respectively. This indicates the potential of garlic nano-emulsion as a robust control tool against this pest. The high efficacy observed against adults is particularly significant, as this life stage is more mobile and poses a greater threat due to its ability to transmit a range of plant viruses. Additionally, treated plants reduced the number of laid eggs, indicating the formulation's potential to disrupt the pest reproductive cycle and further limit population growth. Similarly, a garlic EO formulation containing 0.05% of Triton X-100 and 0.1% dimethyl sulfoxide (DMSO) as an emulsifier demonstrated a significant impact on the ovipositional behavior of *B. tabaci*. Treated leaves exhibited a remarkable 95.55% reduction in eggs laid at a 2% EO concentration (Hussein, 2017). Concerning phytotoxicity, the LD50 induced a non-significant level of damage compared to the control for all four plants throughout the monitoring period. On the other hand, the LD90 exhibited phytotoxicity for cucumber and eggplant despite the emergence of new healthy leaves starting on day 14. Tomato recovered during the monitoring period, with new leaves emerging from the seventh day, while the tested formulations did not significantly affect pepper. Many plant-derived EOs are phytotoxic to plants (Ibrahim et al., 2001; Werrie et al., 2020). However, the extent of plant damage may vary based on several factors, including the concentration or rate applied, the type or the variety of plant, and the plant parts (i.e. leaves and flowers) exposed during spray applications (Cloyd et al., 2009). The phytotoxic effect of EOs can be either a goal or a constraint, depending on their application. In herbicides, phytotoxicity is the desired outcome, as it helps to control unwanted vegetation by inhibiting or damaging plant growth. However, phytotoxicity is a significant constraint for biopesticidal or biostimulation applications. When using biopesticides like EOs or other natural

compounds to control pests, the goal is to target harmful organisms without causing damage to the crop plants. Similarly, in biostimulants—substances that enhance plant growth—minimizing phytotoxicity is essential to avoid unintended harm. Granting insecticidal activity while reducing adverse effects on crops remains a critical challenge for commercializing these bio-based products (Werrie et al., 2020). Furthermore, high-ratio surfactants reduce the interfacial tension between the dispersed and continuous phases in nano-emulsion production, facilitating the formation of smaller droplets. This aspect is particularly relevant when developing nano-pesticides for field crops. In fact, it is now known that surfactants have a phytotoxic effect (Appah et al., 2020; Falk et al., 1994), so the lower their concentration, the lower the risk of phytotoxicity. In conclusion, this study highlights the potential of garlic EO nano-emulsion as a sustainable alternative to conventional insecticides for managing *B. tabaci*, aligning with the growing shift toward reducing traditional chemical pesticides. EOs offer a promising solution due to their complex chemical compositions and multiple modes of action. They could help overcome insecticide resistance and provide an effective control tool against *B. tabaci* populations. In addition, garlic has been used as a food source for centuries, and recent studies suggest that garlic and/or its constituents may offer a range of therapeutic effects, such as supporting cardiovascular health and inhibiting the growth of various bacteria and fungi, as well as cancer. This historical use and scientific evidence contribute to the perception of garlic EO as a relatively safe substance (De Greef et al., 2021; Tesfaye, 2021). However, Regulatory hurdles, such as the biopesticide approval process, can delay market entry. Furthermore, scaling up production of EO-based insecticides can be costly, limiting their commercial availability compared to traditional chemical insecticides. In this scenario, addressing these challenges is crucial to fully harnessing their potential as sustainable pest control solutions.



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Appendix A. Supplementary data

Table A.1: Results of post-hoc comparisons of the GLM model plant*day*dose. Estimate represents the estimated value of the effect of the predictor on the outcome; a negative value indicates an inverse relationship between the predictor and the dependent variable. SE represents Standard Error and df the Degrees of Freedom. t.ratio is the ratio of the estimate to the standard error (Estimate/SE). Asterisks indicate statistically significant pairwise comparisons (p.values < 0.05).

Contrast 1			Contrast 2							
Plant	Day	Dose	Plant	Day	Dose	estimate	SE	df	t.ratio	p.value
Cucumber	3	ctrl	Cucumber	3	LC_50	-0.05567	0.0465	138	-1198	0.9999
Cucumber	3	ctrl	Cucumber	3	LC_90	-0.32100	0.0465	138	-6910	<.0001 *
Cucumber	3	LC_50	Cucumber	3	LC_90	-0.26533	0.0497	138	-5343	0.0004 *
Cucumber	3	LC_50	Eggplant	3	LC_50	0.04167	0.0465	138	0.897	0.9999
Cucumber	3	LC_50	Pepper	3	LC_50	0.03767	0.0465	138	0.811	0.9999
Cucumber	3	LC_50	Tomato	3	LC_50	-0.00908	0.0465	138	-0.196	0.9999
Cucumber	3	LC_90	Eggplant	3	LC_90	0.28000	0.0497	138	5638	0.0001 *
Cucumber	3	LC_90	Pepper	3	LC_90	0.28325	0.0465	138	6097	<.0001 *
Cucumber	3	LC_90	Tomato	3	LC_90	0.08050	0.0465	138	1733	0.9999
Eggplant	3	ctrl	Eggplant	3	LC_50	-0.01400	0.0430	138	-0.326	0.9999

Eggplant	3	ctrl	Eggplant	3	LC_90	-0.04100	0.0465	138	-0.883	0.9999	
Eggplant	3	LC_50	Eggplant	3	LC_90	-0.02700	0.0465	138	-0.581	0.9999	
Eggplant	3	LC_50	Pepper	3	LC_50	-0.00400	0.0430	138	-0.093	0.9999	
Eggplant	3	LC_90	Pepper	3	LC_90	0.00325	0.0465	138	0.070	0.9999	
Eggplant	3	LC_50	Tomato	3	LC_50	-0.05075	0.0430	138	-1180	0.9999	
Eggplant	3	LC_90	Tomato	3	LC_90	-0.19950	0.0465	138	-4294	0.0235	*
Pepper	3	ctrl	Pepper	3	LC_50	-0.01800	0.0430	138	-0.419	0.9999	
Pepper	3	ctrl	Pepper	3	LC_90	-0.03775	0.0430	138	-0.878	0.9999	
Pepper	3	LC_50	Pepper	3	LC_90	-0.01975	0.0430	138	-0.459	0.9999	
Pepper	3	LC_50	Tomato	3	LC_50	-0.04675	0.0430	138	-1087	0.9999	
Pepper	3	LC_50	Tomato	3	LC_90	-0.22250	0.0430	138	-5173	0.0008	*
Tomato	3	ctrl	Tomato	3	LC_50	-0.06475	0.0430	138	-1505	0.9999	
Tomato	3	ctrl	Tomato	3	LC_90	-0.24050	0.0430	138	-5592	0.0001	*
Tomato	3	LC_50	Tomato	3	LC_90	-0.17575	0.0430	138	-4086	0.0472	*
Cucumber	7	ctrl	Cucumber	7	LC_50	-0.15233	0.0465	138	-3279	0.3882	
Cucumber	7	ctrl	Cucumber	7	LC_90	-0.34625	0.0430	138	-8051	<.0001	*
Cucumber	7	LC_50	Cucumber	7	LC_90	-0.19392	0.0465	138	-4174	0.0354	*
Cucumber	7	LC_50	Eggplant	7	LC_50	0.13833	0.0465	138	2978	0.6281	

Cucumber	7	LC_90	Eggplant	7	LC_90	0.11692	0.0465	138	2517	0.9184	
Cucumber	7	LC_50	Pepper	7	LC_50	0.13433	0.0465	138	2892	0.6960	
Cucumber	7	LC_90	Pepper	7	LC_90	0.31725	0.0430	138	7376	<.0001	*
Cucumber	7	LC_50	Tomato	7	LC_50	0.10058	0.0465	138	2165	0.9905	
Cucumber	7	LC_90	Tomato	7	LC_90	0.22200	0.0430	138	5162	0.0008	*
Eggplant	7	ctrl	Eggplant	7	LC_50	-0.01400	0.0430	138	-0.326	0.9999	
Eggplant	7	ctrl	Eggplant	7	LC_90	-0.22933	0.0465	138	-4937	0.0020	*
Eggplant	7	LC_50	Eggplant	7	LC_90	-0.21533	0.0465	138	-4635	0.0067	*
Eggplant	7	LC_50	Pepper	7	LC_50	-0.00400	0.0430	138	-0.093	0.9999	
Eggplant	7	LC_90	Pepper	7	LC_90	0.20033	0.0465	138	4312	0.0221	*
Eggplant	7	LC_50	Tomato	7	LC_50	-0.03775	0.0430	138	-0.878	0.9999	
Eggplant	7	LC_90	Tomato	7	LC_90	0.10508	0.0465	138	2262	0.9808	
Pepper	7	ctrl	Pepper	7	LC_50	-0.01800	0.0430	138	-0.419	0.9999	
Pepper	7	ctrl	Pepper	7	LC_90	-0.02900	0.0430	138	-0.674	0.9999	
Pepper	7	LC_50	Pepper	7	LC_90	-0.01100	0.0430	138	-0.256	0.9999	
Pepper	7	LC_50	Tomato	7	LC_50	-0.03375	0.0430	138	-0.785	0.9999	
Pepper	7	LC_90	Tomato	7	LC_90	-0.09525	0.0430	138	-2215	0.9862	
Tomato	7	ctrl	Tomato	7	LC_50	-0.05175	0.0430	138	-1203	0.9999	

Tomato	7	ctrl	Tomato	7	LC_90	-0.12425	0.0430	138	-2889	0.6981	
Tomato	7	LC_50	Tomato	7	LC_90	-0.07250	0.0430	138	-1686	0.9999	
Cucumber	14	ctrl	Cucumber	14	LC_50	-0.16267	0.0465	138	-3502	0.2424	
Cucumber	14	ctrl	Cucumber	14	LC_90	-0.26150	0.0430	138	-6080	<.0001	*
Cucumber	14	LC_50	Cucumber	14	LC_90	-0.09883	0.0465	138	-2128	0.9930	
Cucumber	14	LC_50	Eggplant	14	LC_50	0.15067	0.0465	138	3243	0.4151	
Cucumber	14	LC_90	Eggplant	14	LC_90	0.10500	0.0430	138	2441	0.9438	
Cucumber	14	LC_50	Pepper	14	LC_50	0.13342	0.0465	138	2872	0.7110	
Cucumber	14	LC_90	Pepper	14	LC_90	0.21150	0.0430	138	4918	0.0022	*
Cucumber	14	LC_50	Tomato	14	LC_50	0.11392	0.0465	138	2452	0.9406	
Cucumber	14	LC_90	Tomato	14	LC_90	0.16050	0.0430	138	3732	0.1356	
Eggplant	14	ctrl	Eggplant	14	LC_50	-0.01200	0.0430	138	-0.279	0.9999	
Eggplant	14	ctrl	Eggplant	14	LC_90	-0.15650	0.0430	138	-3639	0.1733	
Eggplant	14	LC_50	Eggplant	14	LC_90	-0.14450	0.0430	138	-3360	0.3309	
Eggplant	14	LC_50	Pepper	14	LC_50	-0.01725	0.0430	138	-0.401	0.9999	
Eggplant	14	LC_90	Pepper	14	LC_90	0.10650	0.0430	138	2476	0.9329	
Eggplant	14	LC_50	Tomato	14	LC_50	-0.03675	0.0430	138	-0.854	0.9999	
Eggplant	14	LC_90	Tomato	14	LC_90	0.05550	0.0430	138	1290	0.9999	

Pepper	14	ctrl	Pepper	14	LC_50	-0.02925	0.0430	138	-0.680	0.9999	
Pepper	14	ctrl	Pepper	14	LC_90	-0.05000	0.0430	138	-1163	0.9999	
Pepper	14	LC_50	Pepper	14	LC_90	-0.02075	0.0430	138	-0.482	0.9999	
Pepper	14	LC_50	Tomato	14	LC_50	-0.01950	0.0430	138	-0.453	0.9999	
Tomato	14	ctrl	Tomato	14	LC_50	-0.04875	0.0430	138	-1133	0.9999	
Tomato	14	ctrl	Tomato	14	LC_90	-0.10100	0.0430	138	-2348	0.9667	
Tomato	14	LC_50	Tomato	14	LC_90	-0.05225	0.0430	138	-1215	0.9999	
Cucumber	21	ctrl	Cucumber	21	LC_50	-0.13250	0.0430	138	-3081	0.5443	
Cucumber	21	ctrl	Cucumber	21	LC_90	-0.25300	0.0430	138	-5882	<.0001	*
Cucumber	21	LC_50	Cucumber	21	LC_90	-0.12050	0.0430	138	-2802	0.7622	
Cucumber	21	LC_50	Eggplant	21	LC_50	0.12200	0.0430	138	2837	0.7373	
Cucumber	21	LC_90	Eggplant	21	LC_90	0.10225	0.0430	138	2377	0.9604	
Cucumber	21	LC_50	Pepper	21	LC_50	0.09925	0.0430	138	2308	0.9741	
Cucumber	21	LC_90	Pepper	21	LC_90	0.20850	0.0430	138	4848	0.0029	*
Cucumber	21	LC_50	Tomato	21	LC_50	0.08775	0.0430	138	2040	0.9967	
Cucumber	21	LC_90	Tomato	21	LC_90	0.15900	0.0430	138	3697	0.1489	
Eggplant	21	ctrl	Eggplant	21	LC_50	-0.01050	0.0430	138	-0.244	0.9999	
Eggplant	21	ctrl	Eggplant	21	LC_90	-0.15075	0.0430	138	-3505	0.2404	

Eggplant	21	LC_50	Eggplant	21	LC_90	-0.14025	0.0430	138	-3261	0.4018
Eggplant	21	LC_50	Pepper	21	LC_50	-0.02275	0.0430	138	-0.529	0.9999
Eggplant	21	LC_90	Pepper	21	LC_90	0.10625	0.0430	138	2470	0.9348
Eggplant	21	LC_50	Tomato	21	LC_50	-0.03425	0.0430	138	-0.796	0.9999
Eggplant	21	LC_90	Tomato	21	LC_90	0.05675	0.0430	138	1319	0.9999
Pepper	21	ctrl	Pepper	21	LC_50	-0.03325	0.0430	138	-0.773	0.9999
Pepper	21	ctrl	Pepper	21	LC_90	-0.04450	0.0430	138	-1035	0.9999
Pepper	21	LC_50	Pepper	21	LC_90	-0.01125	0.0430	138	-0.262	0.9999
Pepper	21	LC_50	Tomato	21	LC_50	-0.01150	0.0430	138	-0.267	0.9999
Tomato	21	ctrl	Tomato	21	LC_50	-0.04475	0.0430	138	-1040	0.9999
Tomato	21	ctrl	Tomato	21	LC_90	-0.09400	0.0430	138	-2186	0.9889
Tomato	21	LC_50	Tomato	21	LC_90	-0.04925	0.0430	138	-1145	0.9999

VI. General discussion and conclusion

Horticultural crops, encompassing a wide array of fruits, vegetables, and ornamentals, are particularly vulnerable to damage inflicted by phytophagous insects. These insects, which feed on plant tissues, represent a major and persistent threat to crop productivity and quality, impacting both small-scale and large-scale agricultural operations. The ramifications of such damage extend beyond mere aesthetic concerns, significantly reducing crop yields. Losses can range from a substantial 20% to 50% of potential harvests, and in extreme cases of severe infestation, these losses can escalate to a devastating 100% of production (Roditakis et al., 2018). Beyond the quantitative reduction in yield, infestation can also profoundly compromise the qualitative attributes of the product. This includes alterations to its appearance, such as blemishes or deformities, and reductions in its nutritional content, affecting the levels of essential vitamins and minerals. These qualitative changes make the crop products less attractive to consumers, diminishing its market value and impacting the economic viability of horticultural enterprises. Traditionally, farmers have relied heavily on the use of synthetic chemical insecticides to control infestations, with the primary aim of rapidly eliminating harmful insect populations and safeguarding crop yields. This approach, while often effective in the short term, has been characterized by intensive and often indiscriminate application of these products. However, the widespread and prolonged use of chemical insecticides has raised significant environmental and human health concerns. Environmental issues include contamination of soil and water resources, disruption of ecosystem balance, and adverse effects on non-target organisms. Human health concerns range from acute toxicity to chronic health problems linked to pesticide exposure. These concerns have collectively led to an increasing and urgent search for safer and more sustainable alternatives to conventional chemical pest control.

A crucial and increasingly problematic issue associated with the continued use of chemical insecticides is the rapid development of resistance in insect populations. The repeated and often exclusive application of these compounds has exerted strong selective pressure, favoring the survival and reproduction of insects with inherent resistance mechanisms. This process has led to the emergence of increasingly resistant insect populations, rendering traditional chemical treatments less effective over time. Consequently, farmers are often compelled to resort to ever-higher doses of existing insecticides or to switch to more potent and often more toxic chemicals, exacerbating the potentially harmful side effects for the environment and human health (Guedes et al., 2019). The economic implications of this resistance are substantial, increasing the cost of pest management and potentially leading to crop failures.

Amidst the growing need for sustainable pest management solutions, essential oils (EOs) are emerging as a highly promising ecological alternative to synthetic insecticides. EOs, derived from various plant species, offer a range of advantages, including low environmental persistence (Regnault-Roger et al., 2012). Unlike synthetic pesticides that can persist in the environment for extended periods, EOs degrade relatively quickly, minimizing long-term environmental contamination. Furthermore, EOs exhibit reduced toxicity to non-target organisms and beneficial insects, such as pollinators and natural predators, which play vital roles in ecosystem balance and biological control. The complex composition of EOs, comprising numerous active compounds with diverse modes of action, also contributes to a lower likelihood of resistance development by insect populations (Isman MB., 2020). Additionally, EOs represent a readily available and renewable natural resource, aligning with the principles of sustainable agriculture. They could be a viable and integral component of integrated pest management (IPM) programs, which emphasize a holistic and ecologically sound approach to pest control.

Research focused on garlic essential oil (EO) nanoformulations for pest management has yielded promising and compelling outcomes, demonstrating their significant potential as sustainable alternatives to synthetic insecticides. These nanoformulations, which enhance the stability and efficacy of EOs, have shown significant insecticidal activity against three major pests of horticultural crops: *Spodoptera littoralis*, *Phthorimaea absoluta*, and *Bemisia tabaci*. In the case of *S. littoralis*, the garlic nanoemulsions effectively reduced larval feeding behavior and induced high mortality rates, showcasing their potent insecticidal properties. Similarly, for *P. absoluta*, the formulations exhibited strong larvicidal effects, effectively controlling larval populations and significantly impacting the pest's intestinal microbiome, revealing an innovative and multifaceted mechanism of action. Against *B. tabaci*, the formulations were most effective on nymphs and adults, with a comparatively lower impact on eggs, highlighting stage-specific efficacy.

Importantly, the formulations exhibited minimal adverse effects on non-target organisms, including the generalist predator *Nesidiocoris tenuis*, whose mortality remained below 20% even at higher doses. This indicates their potential compatibility and integration within integrated pest management (IPM) programs, where the preservation of beneficial insects is paramount. However, the studies also revealed some degree of phytotoxicity at higher concentrations, particularly in sensitive crops such as cucumber and eggplant. This phytotoxicity was more pronounced in the low-pressure nanoemulsions, suggesting that the processing method plays a crucial role in mitigating adverse effects. In contrast, high-pressure formulations demonstrated reduced phytotoxic effects and greater suitability for agricultural application, indicating the importance of optimizing formulation techniques.

This research underscores the ecological and practical benefits of utilizing garlic EO nanoemulsions as botanical insecticides. These formulations effectively address critical issues



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associated with the use of synthetic pesticides, including environmental contamination, the development of insecticide resistance, and harm to beneficial organisms. While the findings are undeniably promising, further studies are imperative to optimize the formulations and minimize phytotoxicity across a broader range of crops and environmental conditions. Field trials and comprehensive evaluations of their effects on broader ecological networks, including pollinators, natural enemies, and soil microorganisms, will be crucial in validating their long-term efficacy and safety.

In conclusion, garlic EO nanoemulsions represent a viable, environmentally friendly, and sustainable alternative to conventional chemical insecticides. They offer a compelling balance between efficacy and safety, aligning with the overarching goals of sustainable agriculture and contributing to a significant reduction in dependence on chemical pesticides. By harnessing the natural insecticidal properties of garlic EO, these nanoformulations pave the way for a more ecologically sound and resilient agricultural future.

VII. References

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