Selection, Optimization and Technical Application of Entomopathogenic Nematodes for the Biological Control of Major Insect Pests on Tomato

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“Knowledge is not what is memorized. Knowledge is what benefits.”

Al-Shafi‘i

For my Family

Contents

List of Abbreviations

AIC	Akaike's Information Criterion
Bt	*Bacillus thuringiensis*
ca	circa
et al	*et alia*, and other
Ed/Eds	editor/editors
cm	Centimeter
CMC	Carboxy-methyl-cellulose
DBM	The diamondback moth
df	Degree of freedom
e.g	*exempli gratia,* for example
EPNs	Entomopathogenic nematodes
EPPO	The European and Mediterranean Plant Protection Organization
ETPC	Egyptian Tomato insect Pest Complex
GLM	Generalized linear model
H	Height
h	Hours
ha	Hectare
HearNPV	*Heliothis armigera* nucleopolyhedrovirus
IJ	Infective juvenile
IGRs	Insect growth regulators
IPM	Integrated pest management
JKI	Julius Kühn Institute
L	Length
L3	The third instar larva
LC_{50}	Median lethal concentration
LCL	Lower 95% confidence limit
LRT	Likelihood-Ratio-Test
min	Minutes

μl	microliter
μm	micrometer
ml	Milliliter
mm	Millimeter
MRLs	Maximum residue levels
PHI	Pre-harvest interval
RH	Relative Humidity
SD	Standard deviation
TCV	The Tomato chlorosis virus
TYLCV	The Tomato yellow leaf curl virus
UCL	Upper 95% confidence limit
UV	Ultraviolet radiation
W	Width

Summary

Tomato is one of the most important crops worldwide. In Egypt, it occupies the largest cultivated area of vegetable crops. There are numerous insect pests attacking tomato crops in Egypt and they cause significant yield loss. The main tomato insect pests in Egypt are the Egyptian cotton leafworm *Spodoptera littoralis* (*Boisduval*), the black cutworm *Agrotis ipsilon* (Hufnagel), the cotton bollworm *Helicoverpa armigera* (*Hübner*), the Tomato leaf miner, *Tuta absoluta* (Meyrick), and the whitefly *Bemisia tabaci* (Gennadius). The mentioned insect species form a widespread complex which commonly attacks tomato crop in open fields and greenhouses, often more or less concurrently. Nowadays, the main control strategies are based on chemical insecticides with all known negative effects. Therefore, this thesis aimed to introduce a biological control system based on Entomopathogenic nematodes (EPNs) to be utilized against the "Egyptian Tomato insect Pest Complex" (ETPC) in order to have an environmentally sound alternative.

The work started by screening 15 EPN isolates against the four lepidopteran pests (Chapter II). The objective of the performed screening was to find EPN isolates able to infect all ETPC effectively. The target insect species have many similarities such as their feeding behavior, the destructive stage, and the time of attack. Therefore, standard sand bioassays were performed at four doses against larvae of the target species. Based on the obtained results, the most virulent isolates were *Steinernema carpocapsae* BA2, *S. feltiae* Sf, *S. abbasi* abb, and *S. carpocapsae* J7. The second step was testing the efficacy of the selected isolates against the whitefly *B. tabaci* (Chapter III). The isolate *S. feltiae* Sf was the most efficient one against 2nd nymphal instars of the whitefly.

The efficacy of the selected four EPN isolates was tested against *T. absoluta* in sand and tomato leaf bioassays (Chapter IV). *T. absoluta* was selected as the key target among ETPC because of its economic importance. Limited differences were recognised among the isolates when exposed directly in the sand bioassay. In leaf bioassays, all the tested isolates were capable of attacking *T. absoluta* larvae inside and outside the mines. The calculated LC_{50} values were 44 IJs/ml for *S. carpocapsae* BA2, 82 IJs/ml for *S. abbasi* abb, 103 IJs/ml for *S. carpocapsae* J7, and 112 IJs/ml for *S. feltiae* Sf.

The next step was to find suitable EPN concentrations and adjuvants to increase their efficacy against *T. absoluta* larvae on tomato plants (Chapter V). The four isolates were applied at several concentrations and sprayed once or twice within 24 h on infested tomato plants. Applying the nematode suspensions twice resulted in significantly higher mortalities of *T. absoluta* larvae than sprayed once with double concentration. Except *S. abbasi* abb, EPN isolates were able to cause high larval mortality. When different formulations of *S. carpocapsae* BA2 were tested, the adjuvants Xanthan, Nemaperfect®, or Chitosan resulted in a significant increase in the larval mortality. These three adjuvants increased mortality from 70% (water) up to 88% (Xanthan). The adjuvant Nemaperfect® delayed nematode sedimentation in the suspension for about one hour.

In greenhouse experiments, the four EPN isolates were applied twice within 24 h at 5000 IJs/ml in 0.3% Nemaperfect® as an adjuvant on tomato plants infested by *T. absoluta* larvae (Chapter VI). The highest larval mortality was achieved with the isolate *S. carpocapsae* BA2. There were no significant differences among *S. carpocapsae* BA2 (85.5%), *S. feltiae* Sf

(80.5%), and *S. carpocapsae* J7 (76%), whereas *S. abbasi* abb resulted in significant lower mortality (18%).

Based on the results of this extensive stepwise test program, it was possible to develop a biocontrol system against ETPC based on EPNs. Thus, the purpose of the thesis was achieved. The proposed system consists of *S. carpocapsae* BA2, *S. feltiae* Sf, or *S. carpocapsae* J7 in a concentration of 5000 IJs/ml with 0.3% Nemaperfect® or Xanthan. The application of the previous formulation twice within 24 h at dusk or at late afternoon could control *T. absoluta* and the other ETPC effectively. The next step should be the introduction and validation of this method in the practice of Egyptian tomato cultivation.

Zusammenfassung

Die Tomate ist eine der wichtigsten Nutzpflanzen weltweit. In Ägypten erreicht sie die größte Anbaufläche im Gemüsebau. Es gibt zahlreiche Schadinsekten, die Tomatenkulturen in Ägypten befallen und erhebliche Ertragseinbußen verursachen. Die wichtigsten Tomatenschädlinge in Ägypten sind der ägyptische Baumwollwurm *Spodoptera littoralis* (Boisduval), die Ypsiloneule *Agrotis ipsilon* (Hufnagel), der Baumwoll-Kapselwurm *Helicoverpa armigera* (Hübner), die Tomatenminiermotte *Tuta absoluta* (Meyrick) und die Weiße Fliege *Bemisia tabaci* (Gennadius). Die genannten Insektenarten bilden eine weit verbreitete Gemeinschaft, die häufig Tomatenkulturen im Freiland und in Gewächshäusern, oft mehr oder weniger gleichzeitig, befällt. Heutzutage basieren die wichtigsten Bekämpfungsstrategien auf chemischen Insektiziden mit allen möglichen negativen Auswirkungen. Ziel dieser Arbeit ist es daher, ein biologisches Kontrollsystem auf Basis von entomopathogenen Nematoden (EPNs) zu entwickeln, welches gegen alle ägyptischen Tomatenschädlinge eingesetzt werden kann, um den intensiven Einsatz von chemischen Insektiziden in Tomatenkulturen zu reduzieren.

Die Arbeit begann mit der Testung von 15 EPN-Isolaten auf ihre Wirkung gegenüber den vier Schädlingen aus der Ordnung Lepidoptera (Kapitel II). Das Ziel des durchgeführten Screenings war es, EPN-Isolate zu finden, die in gleicher Weise diese Schädlinge effektiv infizieren. Die Zielinsekten haben viele Gemeinsamkeiten, wie z. B. ihr Fressverhalten, das schädliche Entwicklungsstadium und den Zeitpunkt des Angriffs. Daher wurden standardisierte Sand-Bioassays mit vier verschiedenen EPN-Dosierungen gegen Larven der Zielarten durchgeführt. Basierend auf den Ergebnissen waren die virulentesten Isolate *Steinernema carpocapsae* BA2, *S. feltiae* Sf, *S. abbasi* abb, und *S. carpocapsae* J7. Der zweite Schritt war die Prüfung der Wirksamkeit der ausgewählten Isolate gegen die Weiße Fliege *B. tabaci* (Kapitel III). Das Isolat *S. feltiae* Sf war das effizienteste gegen das zweite Nymphenstadium dieses Schädlings.

Die Wirksamkeit der ausgewählten vier EPN-Isolate wurde gegen den Hauptschädling *T. absoluta* in Sand- und Blatt-Bioassays getestet (Kapitel IV). Bei direkter Exposition im Sand-Bioassay waren nur geringe Unterschiede zwischen den Isolaten festzustellen. In Blatt-Bioassays waren alle getesteten Isolate in der Lage, *T. absoluta*-Larven innerhalb und außerhalb der Blattminen zu finden und zu infizieren. Die berechneten LC_{50}-Werte waren 44 IJs/ml für *S. carpocapsae* BA2, 82 IJs/ml für *S. abbasi* abb, 103 IJs/ml für *S. carpocapsae* J7, und 112 IJs/ml für *S. feltiae* Sf.

Weiterhin wurde die Wirksamkeit der ausgewählten Nematodenisolate gegen *T. absoluta*-Larven auf Tomatenpflanzen bewertet (Kapitel V). Die Versuche wurden durchgeführt, um geeignete EPN-Konzentrationen auszuwählen und um mögliche Hilfsstoffe zur Verbesserung der Nematodenwirksamkeit zu testen. Die vier Isolate wurden in verschiedenen Konzentrationen angewendet und ein- oder zweimal innerhalb von 24 Stunden auf befallene Tomatenpflanzen gespritzt. Die zweimalige Applikation der Nematodensuspensionen führte zu signifikant höheren Mortalitäten der *T. absoluta*-Larven als die einmalige Besprühung mit doppelter Konzentration. Außer *S. abbasi* abb waren alle EPN-Isolate in der Lage, eine hohe Larvenmortalität zu verursachen. Darüber hinaus bewirkte eine Formulierung von *S. carpocapsae* BA2 mit den Adjuvantien Xanthan, Nemaperfect® oder Chitosan zu einer signifikanten Erhöhung der Larvensterblichkeit im Vergleich zur Wasser-

Variante. Diese drei Hilfsstoffe erhöhten die Mortalität von 70% (Wasser) auf bis zu 88% (Xanthan). Der Zusatz von Nemaperfect® verzögerte auch die Sedimentation der Nematoden in der Suspension für etwa eine Stunde.

In Gewächshausversuchen wurden die vier EPN-Isolate zweimal innerhalb von 24 Stunden mit 5000 IJs/ml in 0,3% Nemaperfect® als Hilfsstoff auf Tomatenpflanzen angewendet, die von *T. absoluta*-Larven befallen waren. Die höchste Larvensterblichkeit wurde mit dem Isolat *S. carpocapsae* BA2 erreicht, doch gab es keine signifikanten Unterschiede zwischen *S. carpocapsae* BA2 (85.5%), *S. feltiae* Sf (80.5%), und *S. carpocapsae* J7 (76%). Dagegen bewirkte *S. abbasi* abb eine weitaus geringere Larvensterblichkeit (18%).

Durch diese umfangreichen Untersuchungen wurde das Ziel der Arbeit erreicht, ein auf EPNs basierendes System zur Regulierung der ägyptischen Tomatenschädlinge zu entwickeln. Das vorgeschlagene System besteht aus *S. carpocapsae* BA2, *S. feltiae* Sf, oder *S. carpocapsae* J7 in einer Konzentration von 5000 IJs/ml mit 0,3% Nemaperfect® oder Xanthan. Die Anwendung dieser EPN Formulierung zweimal innerhalb von 24 Stunden in der Abenddämmerung oder am späten Nachmittag könnte *T. absoluta* und die anderen Schadinsekten erfolgreich kontrollieren. Der nächste Schritt wäre nun, das Verfahren in Freilandversuchen unter Praxisbedingungen zu testen.

Aim of this thesis

The aim of this work is to develop a foliar application system based on entomopathogenic nematodes (EPNs) against the Egyptian tomato insect pest complex (ETPC). The desired outcome is to introduce these biological control agents as alternatives to replace or reduce using chemical insecticides in tomato pest management.

Chapter I: General introduction

I.1. Status of tomato as crop in Egypt

Tomatoes (*Solanum lycopersicum* L.) are cultivated and consumed in many countries, thus belonging to the most important vegetable worldwide. In Egypt, tomato is the vegetable with the largest extension of crop area (ca. 200,000 ha) making up 28% of the total vegetable area every year. In 2017, Egypt ranked fifth in world tomato production with a yearly yield of 6.7 million tons (Fig. I.1) (FAOSTAT, 2018). The major portion of the production is for local consumption, whereas only around 200,000 tons are exported every year. Most of the Egyptian growers own small fields between 5-30 acres (approximately 2-12 ha) and their income depends mainly on the yield of the cultivated crops in this area. These growers cultivate tomatoes in open fields during spring and summer. Some growers may own larger areas with more than 50 acres. They cultivate tomatoes also in open fields and additionally during winter in greenhouses.

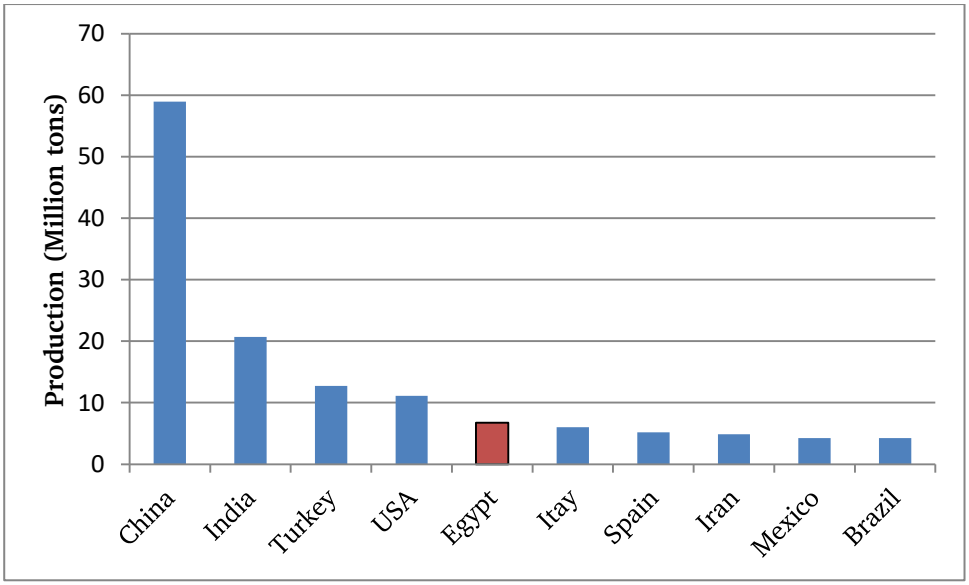


Figure I.1: Rank of "top-ten country" production of tomatoes around the world according to FAOSTAT (2018).

I.2. Key pests on tomato in Egypt

The cultivation of tomatoes in Egypt is challenged by the occurrence of a wide spectrum of insect pests attacking different parts of the plant. Feeding damage by caterpillars or plant-sucking pests can be found on leaves, stems, and fruits. Due to favorable weather conditions in Egypt, most pest species can develop several generations and occur during the whole tomato production season. Native pests of economic importance are the Egyptian cotton leafworm *Spodoptera littoralis* (Boisduval), the black cutworm *Agrotis ipsilon* (Hufnagel), and the cotton bollworm *Helicoverpa armigera* (Hübner) which all belong to the family Noctuidae of Lepidoptera. In recent years, also the invasive Tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera, Gelechiidae), and the whitefly *Bemisia tabaci* (Gennadius) (Hemiptera, Aleyrodidae) cause massive problems. These species belong to the most serious pests which currently cause frequent control interventions, mainly based on chemical insecticides.

I.2.1. The Egyptian cotton leafworm *Spodoptera littoralis*

The Egyptian cotton leafworm, *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae), is a polyphagous insect pest. This pest is native to Africa but also occurs in many countries in southern Europe such as Spain and Italy (Lopez-Vaamonde, 2009). The female moths deposit 20-1000 eggs in clusters on the low surface of leaves (Fig. I.2) (Khalifa et al., 1982). After hatching, larvae feed and develop through six larval instars within about 14 days, and then pupate in the soil at depths of 2 to 5 cm. The larvae climb up the plants and feed on leaves during the night while hiding in the ground or between the plants during the day. They feed on leaves and bore into the fruits as well. Feeding of the last larval instars (from 4th instar on) causes significant losses in the crops and the damage becomes economically important. In Egypt, this pest attacks tomato crops in greenhouses during early spring and from May to October in the open fields.

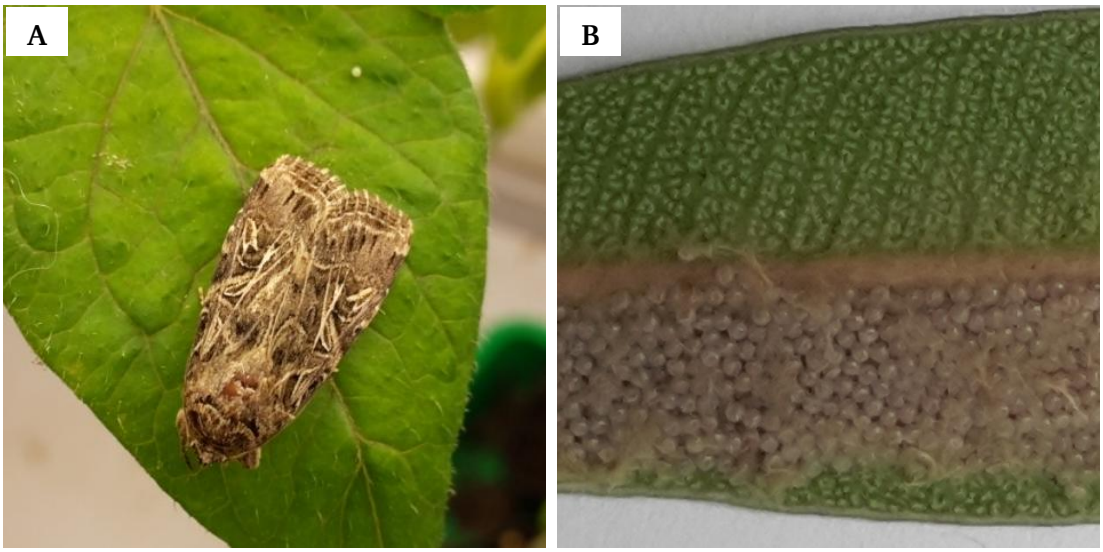


Figure I.2: The Egyptian cotton leafworm, *Spodoptera littoralis*. Adult moth (A), and the eggs (B).

I.2.2. The black cutworm *Agrotis ipsilon* (Hufnagel)

The black cutworm *A. ipsilon* (Hufnagel) (Lepidoptera: Noctuidae) is a serious polyphagous pest that attacks various economic crops in many countries. It is mostly present in every country worldwide (Binning et al., 2015). This pest could infest different crops, such as corn, soybean, cotton, strawberry, potatoes, and tomatoes throughout the year. The insect develops through six larval instars before the pupal stage. The larvae hide during the daylight in the ground and forage during the dark hours. The first three larval instars are able to climb up the plants. Therefore, younger larvae feed on leaves, but older ones attack the plants above the ground surface by cutting the stems off (Fig. I.3). The main crop losses occur because of this feeding behavior, especially during the seedling stage (Showers et al., 1983). This pest has developed resistance against many active ingredients of pesticides worldwide (Yu et al., 2012; Shaurub et al., 2018). Due to the fact that the larvae spend the daylight hours hiding in the soil, regular control strategies are not effective in many cases (Capinera, 2001; Takeda, 2008).

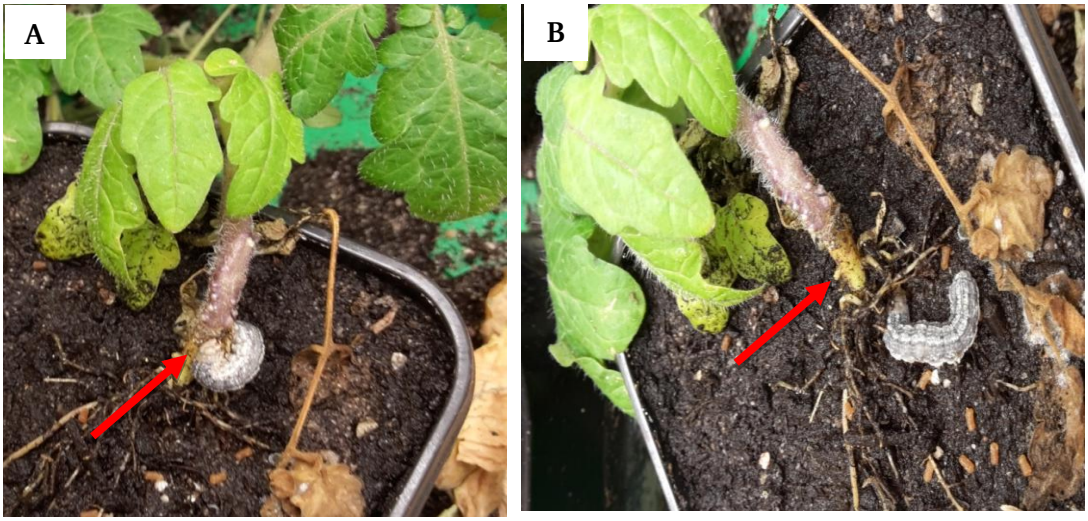


Figure I.3: (A) 6th instar larva of *Agrotis ipsilon* while cutting off tomato seedling near the soil surface. (B) The larval feeding effect on tomato seedling.

I.2.3. The cotton bollworm *Helicoverpa armigera* (Hübner)

The cotton bollworm *H. armigera* (Hübner) (Lepidoptera: Noctuidae) is a highly polyphagous insect pest, damaging a wide variety of economic crops such as tomatoes, cotton, okra, maize, soybeans, and pigeon pea. In tomatoes, *H. armigera* could cause up to 70% yield losses in consequence of fruit boring (Varela et al., 2003). *H. armigera* exists in every continent (Tay et al., 2013; Kriticos et al., 2015). The female moths (Fig. I.4) deposit single eggs on the low surface of the leaves near the plant top. The eggs hatch, and the larvae develop through six larval instars and pupal stage. The larvae occur on the plants usually hidden between leaves, in the flowers, or in the fruits. The insect larvae induce serious losses on tomatoes, as they infest the green fruits preventing the development and consequently causing fruit falling. Larger larvae could attack older fruits as well. This pest attacks tomato crops in Egypt during the period between April and October. The most attractive crop stage for this pest is the flowering and fruiting stage, which is the most critical growing period.

Figure I.4: Adult moth of the cotton bollworm *Helicoverpa armigera*.

I.2.4. The tomato leafminer *Tuta absoluta* (Meyrick)

The tomato leafminer *T. absoluta* (Meyrick) (Lepidoptera: Gelechiidae) is one of the most destructive insect pests of tomato. *T. absoluta* prefers tomato as a host, but it could infest other solanaceous crops such as potatoes, eggplants, and tobaccos (EPPO 2005; Brévault et al., 2014; Mohamed et al., 2015). This insect is native in Peru and is widespread in the entire South America countries. Moreover, *T. absoluta* was detected in Spain in 2006 (Garzia et al., 2012), then it invaded many countries in south and central Europe, northern Africa (Mohamed et al., 2012), and South Asia (Hossain et al., 2016). In Egypt, *T. absoluta* was detected as an invasive pest for the first time in 2009 (Khidr et al., 2013; Goda et al., 2015).

T. absoluta is a serious pest as it completes its lifecycle within one month and has about 12 generations per year in warmer climates (Fig. I.5). The female moths deposit the eggs singly on the leaves or on the stems (Fig. I.6). The emerged larvae pass through four instars before pupation (Silva et al., 2015). The larval stage period takes between 12-20 days depending on the environmental conditions. The last instar larva pupates inside the mine or leaves the galleries and pupates on the leaf surface, between the leaves, on the stems, on the soil surface, or in the soil at a depth of 1-2 cm (Fig. I.7). *T. absoluta* larvae attack tomato plants in all stages from seedling stage to fruiting and harvest stage. The larvae attack the whole plant parts above the ground surface. They prefer the leaves but attack the stems and the fruits as well (Fig. I.8). Larvae mine in leaves and feed on the tissues between the leaf surfaces. The third and fourth larval instars are responsible for the heaviest damage, as they can leave the mine and make new ones in another site on the same leaf or on another one. They also bore in the stems feeding internally. Their attack on fruits, either immature or mature, with boring and feeding the fruit inside is especially serious. This feeding behavior leads to holes in the fruits and subsequently fruit dropping.

Nowadays, *T. absoluta* is the most destructive insect pest on the tomato crop in Egypt since its first detection in the year 2009 (Khidr et al., 2013; Goda et al., 2015). It infests tomato crop grown in open fields during spring and summer, followed by the attack of the tomato plants in greenhouses during winter. The impact of the pest comprises yield reduction up to 100% loss, boosting chemical insecticide applications, and subsequently, raising tomato production costs, prohibiting tomato exportation, and increasing tomato price. No adapted native and effective natural enemies occur in the invaded regions and therefore population growth is rapid and not controlled. Tomato production in Egypt peaked in 2009, but gradually started to decline thereafter probably due to negative effects after the invasion of *T. absoluta* (Fig. I.9). New control methods, safe to the environment, producers, and consumers are urgently needed to overcome this big drawback in efficient and profitable tomato cultivation for Egyptian farmers.

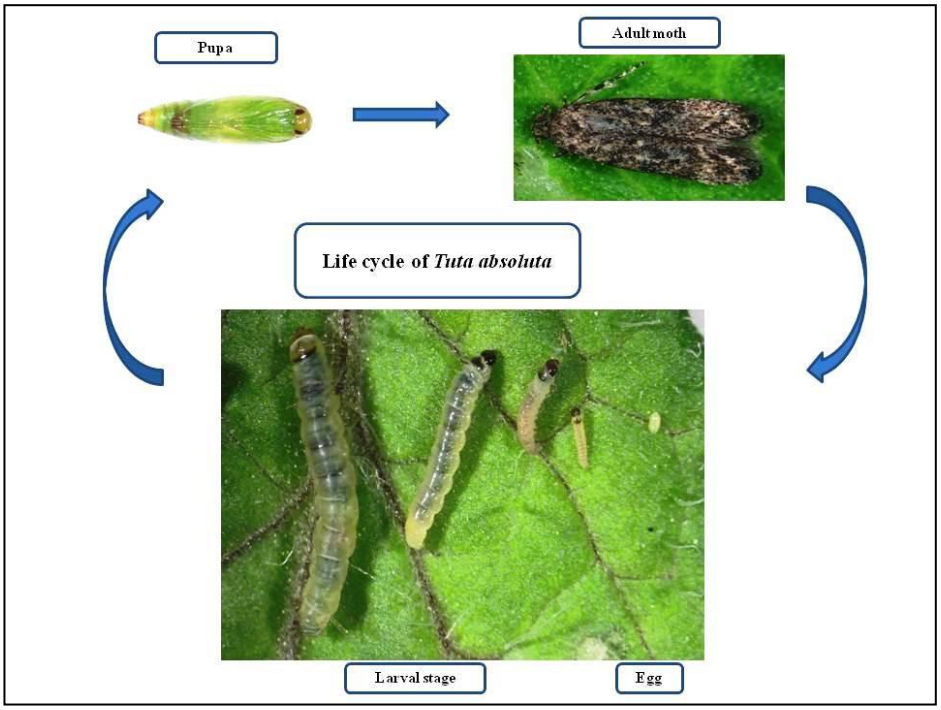


Figure I.5: Lifecycle of the tomato leaf miners, *Tuta absoluta*. Body length: egg=0.2-0.4 mm, larva=0.5-7.5 mm, Pupa=5-6 mm, adult=6-7 mm.

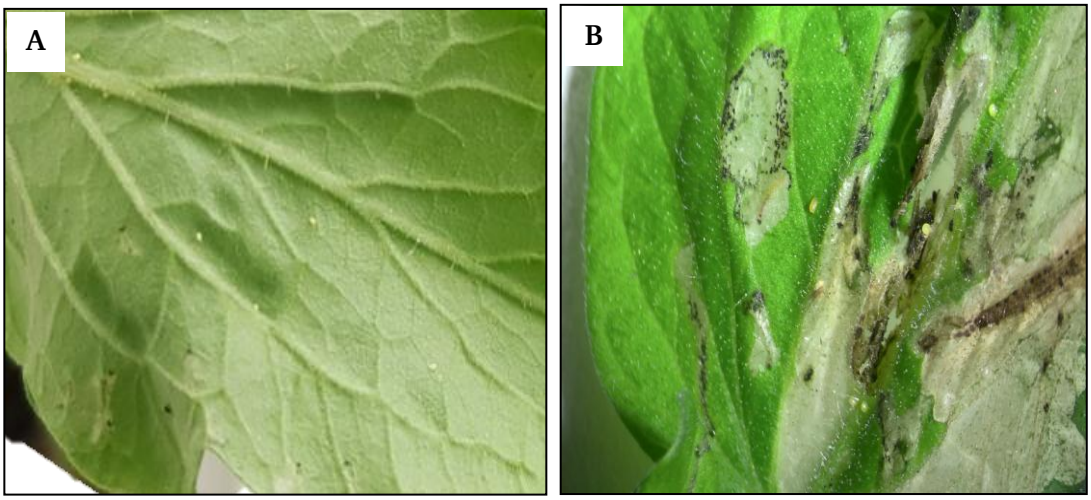


Figure I.6: Eggs of *Tuta absoluta* on both tomato leaf sides. (A) On lower leaf surface. (B) On upper leaf surface.

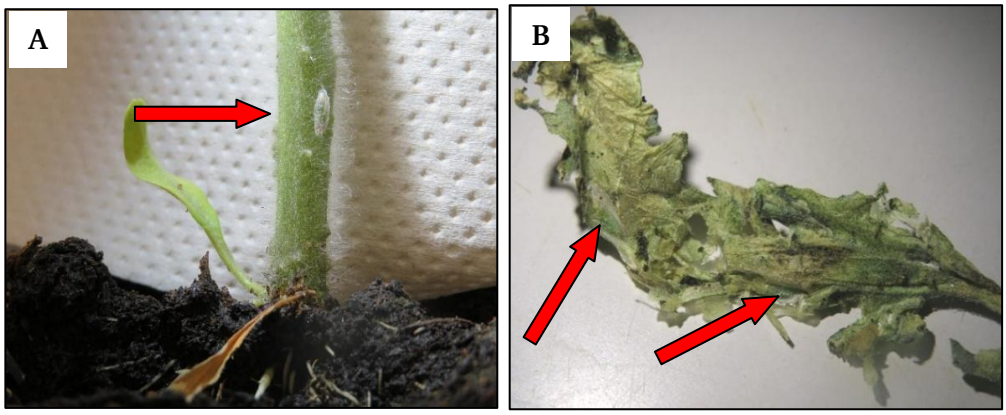


Figure I.7: Pupae of *Tuta absoluta*. (A) On the plant stem near the soil surface. (B) On tomato leaf.

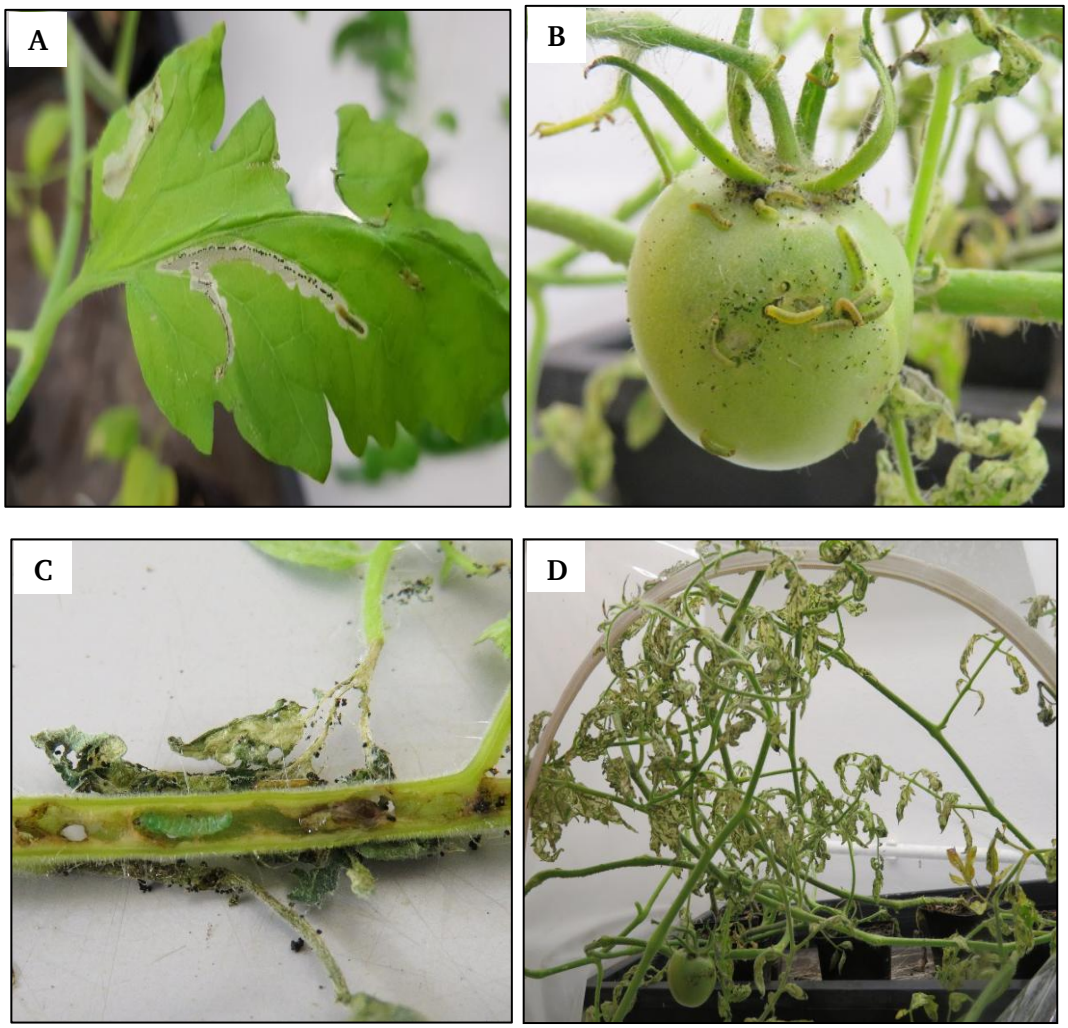


Figure I.8: The damage effects of *Tuta absoluta* larvae on different tomato plant parts. (A) On leaf, (B) On fruit, (C) On stem, (D) on the whole plant.

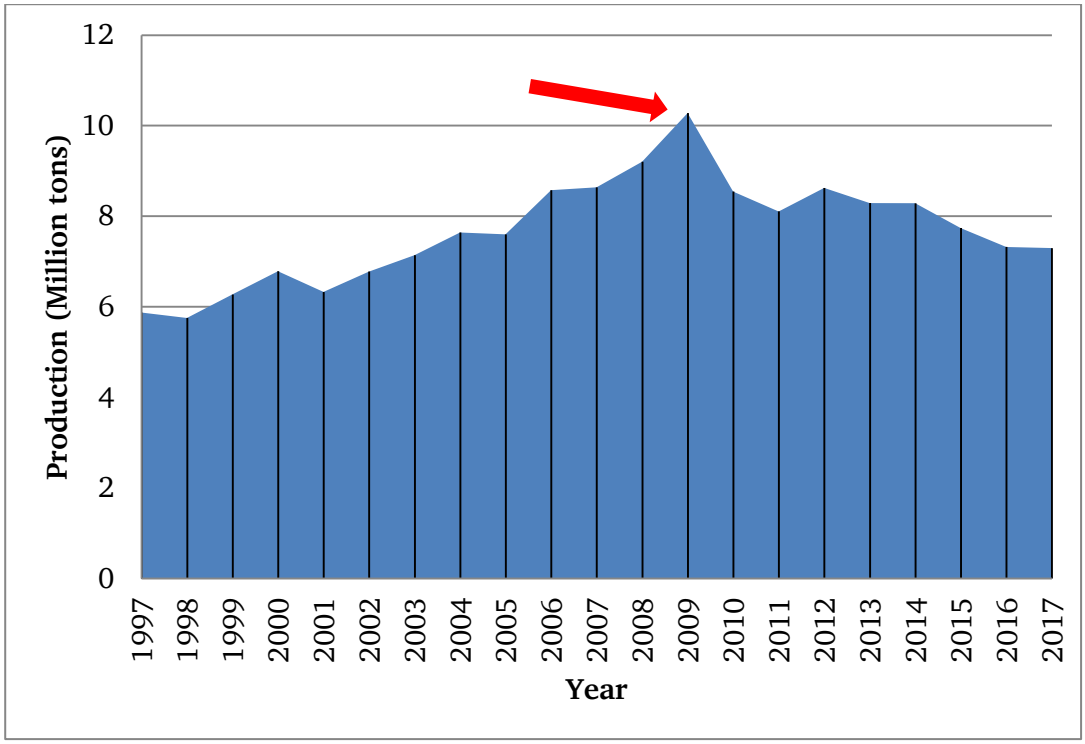


Figure I.9: Tomato production quantity in Egypt between 1997 and 2017 according to FAOSTAT, peaked in 2009 and decreased after the invasion of *Tuta absoluta*.

I.2.5. The tobacco whitefly, *Bemisia tabaci* (Gennadius)

The tobacco whitefly, *B. tabaci* (Gennadius) (Hemiptera: Aleyrodidae), is a pest on many important crops such as cotton, potatoes, sweet potatoes, and tomatoes in many countries. In Egypt, *B. tabaci* is a serious pest on tomatoes. It attacks the crop either in greenhouses or in open fields from day first to harvest. The most serious attack occurs during the summer period. Females lay eggs on the leaves undersides (Fig. I.10). Eggs hatch to the first nymphal instar, which is called "crawler" because it is the only mobile nymphal instar. It moves to search for an appropriate feeding location and afterwards moults to the second nymphal instar, which is immobile and sessile on the leaf underside. Each instar of the first three nymphal instars develops within 2-4 days. The fourth nymphal instar is called pupa, which develops within 6-7 days to the adult fly.

B. tabaci causes crop damages by feeding and by transmitting plant pathogenic viruses (Brown, 2010). Nymphs and adults locate on the lower leaf surface and feed by sucking the sap. Their feeding results in yellow spots which appear on the upper leaf surface. The yellowing increases with infestation time. Also, the feeding stages produce honeydew excretions that cover the leaf surface and cause reduced photosynthesis and growth of sooty mold fungi growth on the leaves and the fruits as well (Stansly and Natwick, 2010). Moreover, *B. tabaci* can transmit some plant pathogen viruses such as Tomato chlorosis virus (TCV) and Tomato yellow leaf curl virus (TYLCV). These viruses result in leaf curling, mosaics, or yellowing in the infested crops (Czosnek and Laterrot, 1997; Gorovits et al., 2013).

The main control programs are based on chemical insecticides. The comprehensive use of chemical insecticides against *B. tabaci* caused resistance of the pest to all active ingredients of

insecticides that have been utilized against it (Ahmad et al., 2002; Wang et al., 2010). Its ability to develop resistance is a frequent problem, even with newly developed products such as insect growth regulators (IGRs) and neonicotinoids (Dennehy et al., 2010). Therefore, methods based on biological control agents should be introduced and integrated into the control programs against *B. tabaci*.

Figure I.10: Adults and nymphs of the whitefly *Bemisia tabaci* sucking the sap on the lower surface of a tomato leaf. Adult length=0.95-1.12 mm.

I.3. Current management methods of the Egyptian Tomato insect Pest Complex (ETPC)

The mentioned key tomato pests form a widespread complex which commonly attacks tomato crop in open fields and greenhouses in Egypt, often more or less simultaneously. Therefore, the growers apply chemical insecticides regularly to control the pests attacking their basic income source. However, the use of chemical insecticides causes negative consequences such as water and soil pollution or negative effects on food chains (Wood and Ehui, 2005). Furthermore, their frequent use leads to resistance development in insects (Van Bortel et al., 2008). As pests become more and more resistant to many active ingredients, farmers use pesticides more frequently and with higher application rates than recommended. As a result, the risk of excessive pesticide residues on the crop increases, making the tomato fruit unsafe for consumers and not suitable for exportation. Thus, farmers need pest management solutions against this “Egyptian Tomato insect Pest Complex” (ETPC) because there is no single problem, but many. Using insecticides is the choice between the devil and the deep blue sea - applying active ingredients with broad-spectrum does not follow principles of integrated production and cause negative side effects on natural enemies and the environment; applying many selective ones raises costs and probably also pesticide residues on the crop.

Pest management relying on biological control methods can be effective, but more sustainable solution (Lacey et al., 2006). Biological control refers to the utilization of antagonists to reduce the pest population. Methods based on microbial or macrobial biocontrol agents have become alternatives with a steadily growing market (van Lenteren et al., 2018). Biological control agents against pest insects function as predators, parasitoids, or pathogens. Predators, such as *Macrolophus pygmaeus* (Rambur) can consume a large number

of *T. absoluta* eggs and the whitefly nymphs. Parasitoids like *Trichogramma* spp. parasite eggs of *H. armigera* (El-Wakeil, 2007; El-Heneidy and El-Dawwi 2010) and *T. absoluta* (Schäfer and Herz, 2020). Also, the parasitic wasp *Encarsia formosa* Gahan (Hymenoptera, Aphelinidae) can parasite both the tobacco whitefly *B. tabaci* and the greenhouse whitefly *Trialeurodes vaporariorum* (Westwood) (Gerling et al., 2001). Pathogens include viruses, bacteria, and fungi. They kill their host by causing disease. Insect pathogenic bacteria such as *Bacillus thuringiensis* (Bt) can be used against *T. absoluta* (González-Cabrera et al., 2011; Alsaedi et al., 2017; Jamshidnia et al., 2018), *S. littoralis* (Abdelkefi-Mesrati et al., 2011), and *A. ipsilon* (Yan et al., 2020). Insect viruses are available to control *A. ipsilon* (El-Salamouny et al., 2003), *H. armigera* (Eroğlu et al., 2019), *T. absoluta* (Gómez Valderrama et al., 2018; Ben Tiba et al., 2019). Fungi like *Beauveria bassiana* are applied against *B. tabaci* (Islam et al., 2010). The applications of these pathogens require adequate formulation to be effective and need particular registration procedures as plant protection products in most countries. On the other hand, entomopathogenic nematodes (EPN) are macrobials carrying symbiotic bacteria which cause disease and death of host insects after infection. Nematodes can be considered as the carrier for these pathogens. EPNs were reported to be effective against *S. littoralis* and *A. ipsilon* (Abonaem, 2013), *H. armigera* (Kary et al., 2012), *T. absoluta* (Batalla-Carrera et al., 2010), and *B. tabaci* (Cuthbertson et al., 2007a,b).

Biological control has many advantages in comparison with insecticides, even though it is sometimes costly and needs a lot of time and particular knowledge. Biological control agents are environmentally friendly and their use has minor or no adverse consequences on soil, water, and the environment. Another benefit is that these natural enemies do not enhance developing resistance in insects (De Clercq et al., 2011). Also, it is a sustainable control method, if the biological organisms were established successfully in the host insect environment.

Biological control agents are somewhat specific to certain insect groups. For example, baculoviruses are very specific: the baculovirus *Heliothis armigera* nucleopolyhedrovirus (HearNPV) which is active against *H. armigera* is not infective for *T. absoluta*. The same is true for most parasitoids: e.g. the parasitic wasp *E. formosa* can parasite the whiteflies but not the lepidopterans. Among biological control agents, only EPNs can infect a broader range of insects, even from different insect orders. For this reason, EPNs are very promising as antagonists of various tomato insect pests. They are candidates of the first-choice to test against the ETPC as the aim of this work.

I.4. Perspectives for biological control using entomopathogenic nematodes (EPNs) for control of the Egyptian Tomato insect Pest Complex (ETPC)

EPNs are roundworms (Nematoda, Rhabditida) that inhabit the soil and live mutualistically with bacteria. The families Steinernematidae and Heterorhabditidae are associated with bacteria belonging to the genera *Xenorhabdus* and *Photorhabdus,* respectively (Bird and Akhurst, 1983). EPNs have some features which make them potent biocontrol agents. They can kill their hosts fast, often within 48 h (Grewal and Georgis, 1999; Shapiro-Ilan et al., 2014). EPNs can move inside the substrate (usually soil), searching for their hosts. EPNs have a quite broad host range with susceptible hosts among various insect taxa. Vulnerability and susceptibility of hosts are often more dependent on whether EPNs can locate and penetrate them than on taxonomic relatedness. Nowadays, EPNs can be produced *in vivo* and *in vitro* as well, and are commercially available in many countries worldwide (Ehlers, 2001; Grewal et

al., 2005; Peters et al., 2017). Moreover, they are quite easy to apply, and their use does not require any pre-harvest interval (PHI) and is exempted from pesticide registration in many countries. Also, their application is safe for the environment and their effect on non-target organisms in the crop systems is limited.

I.4.1. Biology and life cycle of EPNs

EPNs occur in the soil as infective juveniles (IJs) which are the only free-living stage. IJs are the third juvenile instars that keep the cuticle of the second juvenile instars (Fig. I.11). The extra cuticle works as a sheath to protect IJs during their movement among the soil particles. IJs are the only non-feeding stages; they can survive in the soil without feeding for several months, depending on the stored lipids in their bodies as energy reservoirs (Selvan et al., 1993). In the soil or on plant leaves, they stay waiting (ambushers) or move searching (cruisers) for a suitable insect host (Fig. I.12). The nematode IJs locate their hosts by moving following the insect cues (Lewis et al., 2006). Once they find an insect host, they invade it through the spiracles, the mouth, the anus, or through penetrating the cuticle (only in *Heterorhabditis* spp.). Afterwards, they release the bacterial cells in the insect hemolymph (Martens and Goodrich-Blair, 2005). The bacteria multiply and produce exotoxins and endotoxins that kill the host through septicemia (Akhurst and Boemare, 1990; Dowds and Peters, 2002). Moreover, they produce antimicrobial agents against other contaminants (Boemare et al., 1996). In some cases, nematodes release a substance help to inhibit the host immune resistance (Götz et al., 1981; Griffin et al., 2005). The proteins produced by the bacteria metabolize the cadaver tissues into liquid substances on which the nematodes feed. IJs develop into the fourth juvenile instars and subsequently into adult females or males in the case of *Steinernema* or into hermaphrodites in the case of the first generation of *Heterorhabditis*. The adult females or hermaphrodites lay eggs that hatch into juveniles. The nematode juvenile develops through four instars into an adult. In the second generation, the new juveniles develop into males or females in both nematode genera. The nematodes continue feeding and producing offspring through one or more generations. Once the available food becomes deficient, the third juveniles keep the previous instar cuticle to become IJs and emerge from the insect cadaver searching for new hosts (Poinar, 1990). The number of emerged IJs from one cadaver can be more than 100,000 IJs searching for new insect hosts (Figure I.13).

Figure I.11: Infective juvenile (IJ) of *Steinernema carpocapsae*. Photo taken with Zeiss Axioscope microscope, magnification 100x. IJ length=438-650 µm.

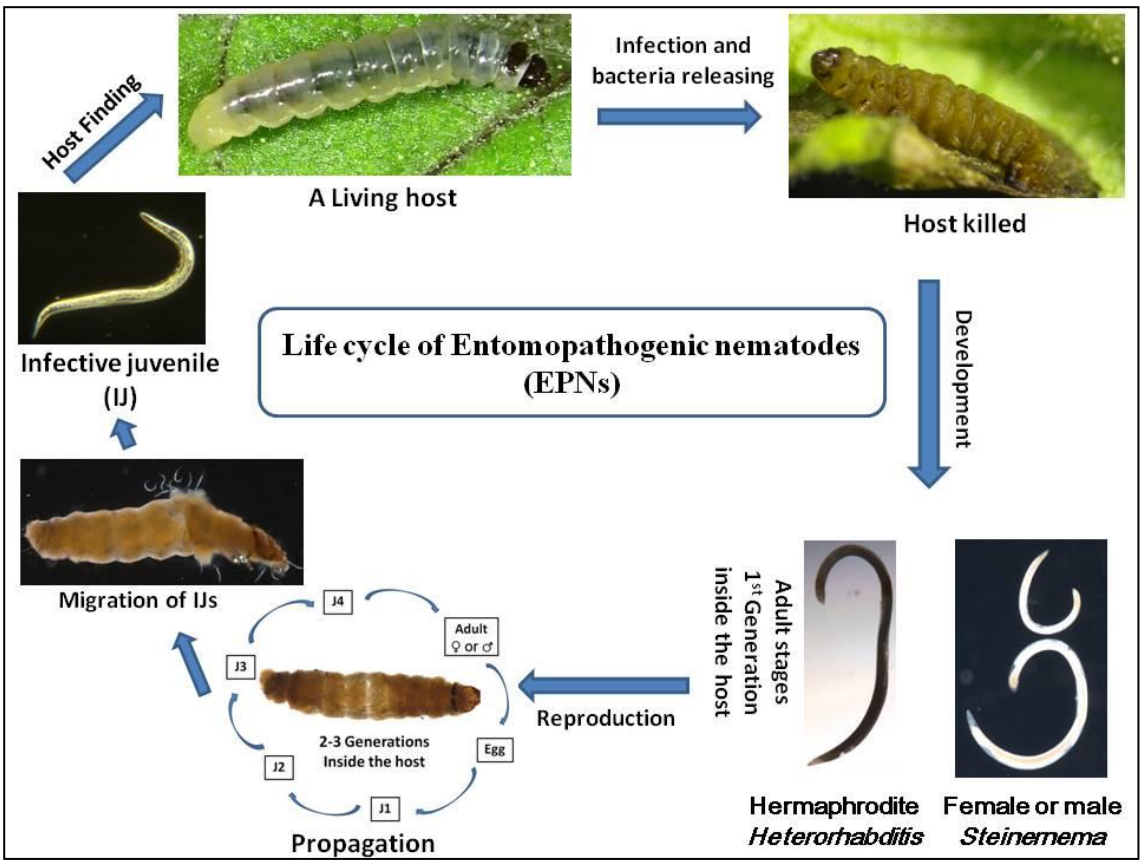


Figure I.12: Lifecycle of entomopathogenic nematodes. Presentation of EPN and host larva on different scales, real sizes account to in case of IJ=0.4-1.5 mm, in case of *Tuta absoluta* 4th instar larva=7.5 mm

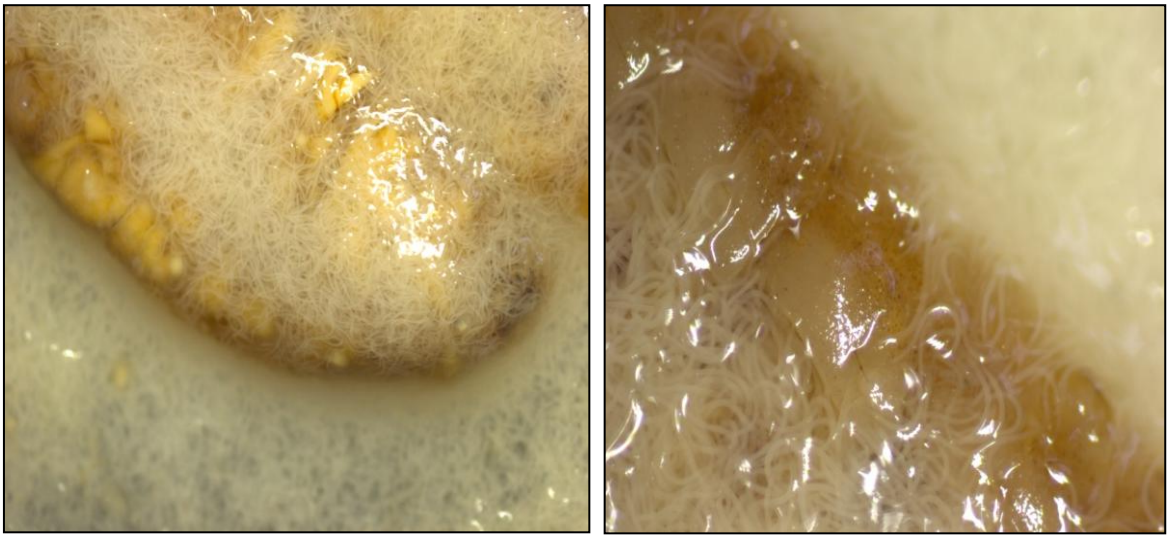


Figure I.13: Entomopathogenic nematode infective juveniles (IJs) migrate from insect host cadaver searching for new hosts. IJ length=438-650 µm.

I.4.2. Status quo of research on EPN against the ETPC

The efficacy of EPNs against insect pests has been proved in the laboratory and open field trials by numerous authors (Grewal et al., 2005; Peters et al., 2017). Although many laboratory bioassays indicated that EPNs can be highly virulent, results from field trials were often variable. Many EPN species have been produced and commercialized to be used against several insect species (Shapiro-Ilan et al., 2014; Peters et al., 2017). Also, the potential efficacy of EPN against target pests forming the ETPC has already been investigated and the results obtained so far are reviewed in the following. EPNs have been used against host insects in soil by different methods, but also against foliar or external feeding insects on plant parts. In general, soil applications have more prospects, because EPNs are primarily soil-dwelling organisms, while above-ground applications remain a challenge. When nematodes are applied to plant leaves, they are confronted with some factors that can impair their effectiveness or viability. The most limiting factors for nematode success are desiccation, ultraviolet radiation (UV), and high temperature (Grewal and Georgis, 1999; Shapiro-Ilan et al., 2006). In some cases, adding adjuvants that work as humectants, surfactants, or adhesives to the nematode suspension reduced the impact of such disturbing factors and increased their efficacy (Schroer et al., 2005).

I.4.2.1. EPNs against noctuid pests

The efficacy of nematodes against *S. littoralis*, *A. ipsilon*, and *H. armigera* was tested in many cases. The most efficient concentration of *Neoaplectana carpocapsae* (=*S. carpocapsae* (Weiser)) was 8000 IJs/ml when tested against all *S. littoralis* larval instars on castor leaves in leaf bioassays (El Kifl, 1980), while another report showed that a *Heterorhabditis* isolate at a dose of 250 IJs/larva obtained 100% larval mortality in *S. littoralis* in laboratory experiments (Shamseldean et al., 2009). The nematode isolates *S. carpocapsae* (All) and *S. carpocapsae* (S2) were reported to be the most efficient ones against *S. littoralis* in cabbage leaf disk bioassays (Salem et al., 2007). The effect of *H. bacteriophora* on *S. littoralis* larvae was higher than that of *S. riobrave* under laboratory conditions (86% and 71% mortality, respectively)

(Shairra and Noah, 2014). The most susceptible larval instars of *S. littoralis* to different dose levels of *S. carpocapsae* were the 3rd and 4th instar larvae (Abdel-Kawy, 1985). In an early study, El Kifl (1984) investigated the factors affecting the efficacy of *Neoaplectana carpocapsae* and *H. heliothidis* Poinar against *S. littoralis* larvae in pot trials. The dose of 18,000 IJs/pot resulted in 100% larval mortality. Under greenhouse conditions, *H. heliothidis* was more virulent than *H. bacteriophora* Poinar against prepupae and pupae of *S. littoralis* (Mogahed and El Kifl, 1991). Under open field conditions, the nematode application ($9*10^4$ IJs/m^2 of soil surface) against 4th instar larvae of *S. littoralis* on corn resulted in 100% larval mortality with *S. carpocapsae* isolate (BA2) and 33.6% with *H. bacteriophora* isolate (S1) (Abonaem, 2013).

EPNs were also highly virulent against *A. ipsilon* in several studies. In laboratory bioassays, application of *S. carpocapsae* (Sc) and *H. bacteriophora* (Hb) at 100 IJs/ml resulted in 100% mortality in larvae and pupae of *A. ipsilon* (Fetoh et al., 2009), and this finding was confirmed by a similar study with another *Heterorhabditis* isolate (Shamseldean et al., 2009). In greenhouse experiments, *S. carpocapsae* applied at a rate of 240 IJs/ml was able to kill 74% of *A. ipsilon* larvae on canola leaves (Mahmoud, 2014). The infection to *A. ipsilon* larvae by EPNs was lower under field conditions. In strawberry fields, the application of *S. carpocapsae* (Sc) and *H. bacteriophora* (Hb) at 2000 IJs/ml resulted in 70% and 80% host mortality (Fetoh et al., 2009). In corn fields, isolates *S. carpocapsae* (BA2) and *H. bacteriophora* (S1) were applied against 4th instar larvae of *A. ipsilon* at a rate of $9*10^4$ IJs/m^2 of the soil surface and achieved 100% and 83.8% larval mortality, respectively (Abonaem, 2013).

Many EPN species were reported to be pathogenic to *H. armigera*, such as *Heterorhabditis* sp., *S. carpocapsae*, *S. riobrave* Cabanillas, Poinar & Raulston (Tahir et al., 1995), and *S. glaseri* (Steiner) (Patel and Vyas, 1995). In an extensive laboratory screening, 27 nematode isolates resulted in larval mortality ranging between 41% and 94% (Seenivasan and Sivakumar, 2014). In other laboratory bioassays, the susceptibility of early instars of *H. armigera* to *H. indica* was higher than that of later instars as indicated by median lethal time values (Divya et al., 2010). The infectivity of *Steinernema* sp. for *H. armigera* pre-pupa was 43% under laboratory conditions (Ali et al., 2007). In pot bioassays, five steinernematid species showed high infectivity against *H. armigera* (Ali et al., 2008). The application of the species *S. masoodi* at a concentration of $6*10^9$ IJs/ha ($6*10^5$ IJs/m^2) resulted in 70% mortality in *H. armigera* on chickpea, *Cicer arietinum* L. (Hussain et al., 2014). Foliar application of *Heterorhabditis* sp. (100,000 IJs/m^2) controlled 32% of *H. armigera* larvae when applied alone and 47% when applied with adjuvants (5% starch mixed with gum Arabic) (Vyas et al., 2002).

I.4.2.2. EPNs against the Tomato Leafminer

Several studies were performed to test EPNs infectivity against *T. absoluta* under laboratory, greenhouse, and even open field conditions after the arrival of this invasive pest in various countries. The performance of isolates of *S. carpocapsae*, *S. feltiae* (Filipjev), and *H. bacteriophora* was examined against *T. absoluta* larvae and pupae under controlled laboratory conditions by Batalla-Carrera et al. (2010). They found that larvae were susceptible, but pupae were resistant. The application of 50 IJs/cm^2 on larvae caused mortality between 86.6% and 100% in Petri dish experiments. The same application on pupae resulted in much lower pupal mortality between 1.7% and 8.3%. Surprisingly, soil application of EPNs against

larvae that invade the soil to pupate led to high mortality rates under laboratory conditions (Garcia-del-Pino et al., 2013). Whereas soil application of *S. carpocapsae* and *S. feltiae* did not cause noticed mortality in the pupae, the juveniles of *S. carpocapsae* caused high mortality in adults emerging from the soil (Garcia-del-Pino et al., 2013).

As *T. absoluta* larvae mine and feed inside galleries on tomato leaves, the nematode ability to infect the larvae in tomato leaves was also investigated. According to these studies, EPNs can infect *T. absoluta* larvae inside their mines. Under laboratory conditions, *S. carpocapsae* and *S. feltiae* obtained high mortality rates in *T. absoluta* larvae inside their mines in tomato leaf disk bioassays (Van Damme et al., 2016). EPNs achieved infection rates between 77.1% and 91.7% in *T. absoluta* larvae in leaf bioassays (Batalla-Carrera et al., 2010). In another leaf bioassay, *S. monticolum* and *H. bacteriophora* (isolate HP88) caused high mortality rates in *T. absoluta* larvae (80-100% mortality) (Shamseldean et al., 2014). The foliar application of *S. feltiae* at 10,000 IJs/ml against *T. absoluta* larvae on tomato leaves was as effective as Spinosad, a natural biopesticide obtained from the bacterial species *Saccharopolyspora spinosa* (Jacobson and Martin, 2011). In contrast, the study by Türköz and Kaskavalci (2016) recorded only 19% larval mortality and 7% pupal mortality when *S. feltiae* was applied against *T. absoluta* larvae inside the mines and pupae.

Under greenhouse conditions, the foliar application of EPN against *T. absoluta* larvae on potted tomato plants obtained larval mortalities of 87% for *S. carpocapsae* and 95% for both *H. bacteriophora* and *S. feltiae* (Batalla-Carrera et al., 2010). Moreover, the application of four EPN species at a rate of 50 IJs/cm^2 against *T. absoluta* larvae on tomato plants in cages resulted in 39.3% to 94.3% larval mortality (Gözel and Kasap, 2015). In contrast, a very low reduction of leaf mines (12.9%) was obtained after an application of *S. carpocapsae* (250 IJs/ml) against *T. absoluta* on tomato plants under greenhouse conditions in Egypt (Sabry et al., 2016).

In open field applications, *T. absoluta* larvae were susceptible to four EPN species, but the degree of larval susceptibility to infection differed among the EPN species (Gözel and Kasap, 2015). Another field application of EPNs against *T. absoluta* larvae resulted in larval mortality ranging between 60% and 80% with *H. bacteriophora* and between 58% and 67% with *S. monticolum* (Shamseldean et al., 2014). Even lower larval mortality (40-50%) was recorded after using *S. feltiae* (1000 IJs/ml) against *T. absoluta* larvae in tomato fields (Jacobson and Martin, 2011).

I.4.2.3. EPNs against the whitefly

Many EPN species were reported to be infective for the whitefly nymphs and adult stages. In general, *S. feltiae* is considered the most effective species against whiteflies, both *B. tabaci* and *T. vaporariorum*. Juveniles of *S. feltiae* were more infective than those of *H. bacteriophora* against adults and second nymphal instars of *T. vaporariorum* on cucumber and pepper in laboratory bioassays. Likewise, in greenhouse trials, *S. feltiae* was more efficient than *H. bacteriophora* as it obtained the same nymphal mortality with lower concentrations (Rezaei et al., 2015). The first three instars of *B. tabaci* were more susceptible to *S. feltiae* application (10,000 IJs/ml) than the fourth instar on tomato plants (Cuthbertson et al., 2003). Nevertheless, adults of the greenhouse whitefly *T. vaporariorum* were susceptible to *S. feltiae* infection (Laznik et al., 2011). Generally, the second nymphal instar of *B. tabaci* was the most

susceptible developmental stage to *S. feltiae* and *S. carpocapsae* infection (Cuthbertson et al., 2007a,b).

Many factors could affect the EPN efficacy against the whitefly, for instance, the surface and other attributes of the plant species on which nematodes are applied. The efficacy of *S. feltiae* (10,000 IJs/ml) was varied when tested against *B. tabaci* on five plant species (Head et al., 2004). Likewise, the EPN efficacy against *T. vaporariorum* on cucumber was significantly higher than on pepper (Rezaei et al., 2015). The authors suggested that the reason behind the significant differences could be the leaf structures such as waxy surfaces or hairs that may affect the nematode movement, which reducing the nematode efficacy. Nevertheless, there were no significant differences among the mortalities in *B. tabaci* when treated by *S. feltiae* on different plant leaf types under the ideal environmental conditions for EPNs activity (Cuthbertson et al., 2007b). Environmental conditions, especially temperature and humidity, are probably more crucial factors affecting the foliar applications of EPNs against *B. tabaci* (Cuthbertson et al., 2007b). Moreover, adding some adjuvants in the foliar application could increase the nematode efficacy against the whitefly. Adding the non-ionic surfactant TritonX-100® to *S. feltiae* suspension increased the mortality in *B. tabaci* on tomato plants to 63%, while adding the surfactant Agral® (Syngenta) resulted in 50% mortality (Head et al., 2004).

I.5. Current research gaps and objectives of the thesis

The Egyptian tomato growers currently possess no alternative solutions to chemical insecticides to combat tomato insect pests, although so many investigations on possible biological control methods were conducted. Among biological control agents, only EPNs may provide a single agent against the whole target ETPC. The review of existing research studies proved that EPNs were promising and could be used against the target pests. EPNs are applied successfully against soil-borne insect pests in many crop systems. However, the economic relevant pest stages within the ETPC mainly attack the leaves, so that it is important to target them on foliage. The development of suitable techniques for EPN application against pests feeding on leaves is much more difficult, especially under Egyptian growing conditions with high temperatures and low humidity. Particular formulations and application schedules are required to maintain EPNs ability to locate and infect host insects under these conditions. Firstly, screening of different nematode species against ETPC is required. Such comprehensive screening was not done in previous studies, and it is not clear which nematode species could effectively attack all target pests of ETPC. In the next step, the most effective EPN species or isolate needs to be further investigated, whether it can also attack the pest in foliar applications. Then, potential formulations and application methods must be optimized and tested under semi-field conditions towards an economic feasible control system for these pests.

The main aim of this work is to develop a foliar application system based on EPNs against the ETPC. The desired outcome is to introduce these biological control agents as alternatives to replace or reduce using chemical insecticides in tomato pest management. To achieve this goal, it is necessary to select and characterize suitable EPN strains in a step-wise experimental design and to elaborate proper application methods for use in the tomato crop system.

To achieve the previous objectives the work was conducted as following (Fig. I.14):

1. Screening strains of various EPN species of different origin for their potential to control the lepidopteran pests of the ETPC. (Chapter II).
2. Test the efficacy of the most efficient isolates against the tomato leaf-sucking pest *B. tabaci* a major insect pest attacking tomato crop in Egypt (Chapter III).
3. Bioassays to investigate the ability of the most efficient isolates according to the previous screening to infect *T. absoluta* larvae as tomato leaf-mining pest in Egypt, on sand bioassay and tomato leaf bioassay (Chapter IV).
4. On plant experiments (Chapter V):
   a. Experiments were conducted to evaluate the efficacy of the selected nematode isolates against *T. absoluta* larvae on tomato plants. This experiment was to find a proper concentration to be used against *T. absoluta* larvae on tomato plants.
   b. Further experiments were performed for screening adjuvants. In this bioassay, the selected concentration based on the previous experiment result was used to select one adjuvant that can increase the nematode efficacy against *T. absoluta* larvae on tomato plants.
5. Greenhouse experiment (Chapter VI):
   The suggested control system was evaluated against *T. absoluta* larvae artificially infested tomatoes under greenhouse conditions in summer. This experiment was conducted to assess the control method under natural conditions and high temperatures as well.

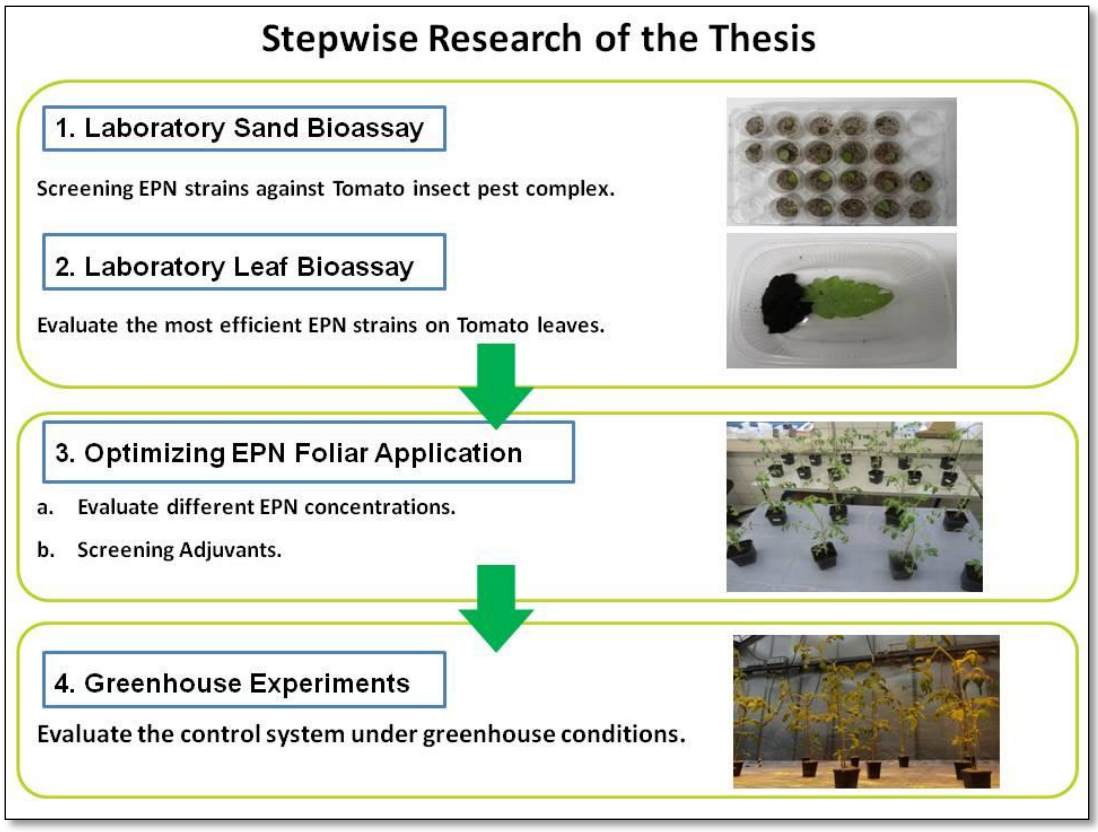


Figure I.14: The schematic representation of the thesis framework.

Chapter II: Laboratory screening of entomopathogenic nematodes for efficacy against lepidopteran pests of the Egyptian Tomato insect Pest Complex

II.1. ABSTRACT

The tomato crop in Egypt is attacked by many insect pests. The most economically damaging pests are lepidopteran species and there are many similarities among them, such as their feeding behavior, the destructive stage, and the attack time. Therefore it is reasonable to introduce one active agent suitable for controlling all these pests. Entomopathogenic nematodes (EPNs) are potent biological control agents with a wide host range, thus being good candidates to control different pests. Screening of 15 EPN isolates was performed against four lepidopteran tomato pests in standard sand bioassays. The comparison among the different isolates was based on their efficacy at four doses against larvae of the respective target species. All tested EPN isolates were able to infect the larvae even at low dose levels. In general, isolates of *Steinernema* were more virulent than those of *Heterorhabditis*. The most efficient isolates within the highest dose (20 IJs/larva) were compared within the lower doses (10 and 5 IJs/larva). According to this evaluation, four EPN isolates were determined as most efficient against the tested insects and were selected to be evaluated in further bioassays. The selected isolates derived all from the genus *Steinernema*, namely *S. carpocapsae* BA2, *S. feltiae* Sf, *S. abbasi* abb, and *S. carpocapsae* J7.

II.2. INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is an important crop and food for people around the world. Also in Egypt, this crop is of crucial economic importance for farmers, who cultivate tomatoes around the year in open fields or greenhouses. But tomato is a target for many insect pests, which cause a significant reduction in yield. The most economic insect pests attacking tomato in Egypt include the tomato leaf miner (*Tuta absoluta* Povolny), the cotton bollworm (*Helicoverpa armigera* (Hübner)), the cotton leafworm (*Spodoptera littoralis* (Boisduval)), the black cutworm (*Agrotis ipsilon* (Hufnagel)), and the whitefly (*Bemisia tabaci* (Gennadius)). The main control method against these pests is the frequent application of chemical insecticides which negatively impacts the safety of farmers, bystanders, and consumers. For this reason, biological control methods should be introduced to substitute chemical pesticides in tomato cultivation as far as possible.

EPNs can control many insect species. Several EPN species are commercially available and have been used successfully against soil-borne insect pests in various crop systems. Also, foliar application of EPNs is possible in special formulations. EPNs have already been tested against the previously mentioned insect pests in many studies (*Tuta absoluta*: Batalla-Carrera et al., 2010, Jacobson and Martin, 2011; Garcia-del-Pino et al., 2013; Shamseldean et al., 2014; Van Damme et al., 2016; Türköz and Kaskavalci, 2016; *Spodoptera littoralis*: El Kifl, 1980, Shamseldean et al., 2009, Salem et al., 2007, Shairra and Noah, 2014; *A. ipsilon*: Fetoh et al., 2009, Shamseldean et al., 2009, Abonaem, 2013; *H. armigera:* Seenivasan and Sivakumar, 2014; Divya et al., 2010, Ali et al., 2007; Tahir et al., 1995; Patel and Vyas, 1995). Most of these studies were conducted under laboratory conditions and included different EPN species such as *Steinernema carpocapsae*, *S. feltiae*, *S. monticolum*, *S. riobrave*, *S. glaseri*,

Heterorhabditis bacteriophora, and *H. indica*. The efficacy of nematode isolates varied among the insect hosts.

The "Egyptian tomato insect pest complex (ETPC)" mentioned above often occurs simultaneously on the crop in Egyptian horticulture and it would be desirable to develop an EPN based treatment package against all these pests. Hence, the identification of EPN isolates that are most efficient against the whole pest complex is the first step towards developing an innovative EPN-based tool for pest management. None of the studies mentioned above have considered such an approach, so the need for it still exists. Consequently, the explicit goal of this investigation was to find EPNs that are highly effective against all species of the ETPC. The screening was started by identifying those candidates from a collection of available isolates that showed the highest performance against the lepidopteran species (Noctuidae and *T. absoluta*). Crop damage caused by lepidopteran species is economically very serious, as the larvae feed on all green parts of the plant, thus preventing photosynthesis and further plant growth. Seedlings are often destroyed completely (especially due to feeding by *A. ipsilon*). Lepidopteran pests also attack the fruits which are the most important part of the plant for the tomato producers. On the other hand, noctuid larvae, in particular, show some similarity in physiological development and state, feeding behavior, and probably also in susceptibility to EPNs, which makes the choice of a common antagonist more likely. Leaf-mining larvae of *T. absoluta,* an invasive species to Egypt, show a completely different feeding pattern and are a particular threat to tomato production in Egypt. Therefore, it was very important that the screening ensures that selected EPN isolates are definitely effective against this pest.

Although the whitefly *B. tabaci* is a very important tomato pest in Egypt, the current screening was limited to lepidopteran pests within the ETPC. This allowed using standard sand bioassays to test different EPN isolates against target pests in a very comparable test design. Sand bioassays are a proven and accepted technique to study direct dose-response interactions between the EPNs and the host insect (Glazer and Lewis, 2000; Grunder et al., 2005). Also, it was proposed to be used as a standard tool for the quality assessment of EPNs (Grewal et al., 2005). In principle, they are based on the incubation of host and EPNs in conditions favorable for the nematodes' survival and host searching behavior (moist sand) in standardized vials of a certain volume, usually cell culture plates (24 cell well plates). By using this type of bioassay, a comparability of the effects of tested EPNs on the different lepidopteran hosts was ensured.

II.3. MATERIALS AND METHODS

II.3.1. Insects

II.3.1.1. Rearing of the greater wax moth, *Galleria mellonella* Linnaeus for nematode production

The greater wax moth was maintained to be used for nematode production "*in vivo*" at the Julius Kühn Institute (JKI) entomological laboratory. *G. mellonella* is a pest of beeswax in beehives and storage (Chandel et al., 2003). The last instar larva of *G. mellonella* has some

biological properties which make it an excellent host for EPN production. The larvae are highly susceptible to nematode infection (Ramarao et al., 2012), and insect cadavers provide high numbers of nematode progeny (Kotchofa and Baimey, 2019). The wax moth itself has a short lifecycle (Jorjão et al., 2018), and easy rearing on different low coast artificial diets is possible (Metwally et al., 2012).

At JKI, the species was permanently mass-reared using an artificial diet that consisted of wheat flour (30%), wheat germ (30%), corn grits (10%), Brewer's yeast (5%), milk powder (5%), honey (5%), and Glycerol (15%). The rearing took place in plastic boxes (25 cm length (L) * 20 cm width (W) * 15 cm height (H)) with lids that had a handmade metal screen covered opening (Fig. II.1). Larvae were provided by 300 g diet in each box and kept in an incubator at 28-30 °C and darkness until pupation. The pupae were transferred to smaller plastic boxes (20 cm L * 15 cm W * 10 cm H) to allow the emergence of moths and later oviposition. The boxes were closed with their lids, and two tissue paper strips were hanged in the lid as oviposition sites (Fig. II.2). The strips were changed every two days. The strips with the deposited eggs were placed in new plastic boxes containing 300 g diet as food for the hatched larvae. The larvae developed through six larval instars. The last instar larvae were collected and stored in an incubator at 10 °C for no longer than two weeks. Afterwards, the larvae were used for producing the nematodes *in vivo,* as described later in this chapter.

Figure II.1: Rearing boxes for *Galleria mellonella* larvae.

Figure II.2: Oviposition boxes for *Galleria mellonella* moths contain two tissue paper strips hanged in the lid as oviposition sites.

II.3.1.2. Rearing Noctuidae species as target pests for bioassays

Pupae of the black cutworm (*Agrotis ipsilon*) were obtained from Andermatt Biocontrol AG, Grossdietwil, Switzerland. The cotton bollworm (*Helicoverpa armigera*) was received from Bayer AG, Leverkusen, Germany, and the cotton leafworm (*Spodoptera littoralis*) was provided by colleagues from the Department of Bioorganic Chemistry, Max Plank Institute for Chemical Ecology, Jena, Germany. These Noctuid moths are not native to Germany and were therefore reared under containment conditions. They were reared following the same procedure.

The larvae were maintained in plastic boxes (20 cm L * 15 cm W * 10 cm H) containing 250 g artificial diet at 22-25 °C and long-day conditions (16 h light: 8 h dark). The diet was prepared as described by Ivaldi Sender (1974) (Table II.1). The formed pupae were moved to oviposition cages consisted of plastic cylinders (10 cm diameter and 30 cm height) covered with a cotton cloth till the moths emerge (Fig. II.3). The moths were provided with pieces of cotton wool moistened with 10% honey solution. Tissue papers were hanged inside the cylinders as oviposition sites. The laid eggs on the tissue papers were moved to new plastic boxes containing a fresh diet. The 3rd instar larvae were used in the bioassays.

Table II.1: Ingredients of the artificial diet used for rearing larvae of *Agrotis ipsilon*, *Spodoptera littoralis*, and *Helicoverpa armigera* according to Ivaldi-Sender (1974).

Ingredient	Amount
Water	2400 ml
Benzoic acid in alcohol	9 g
Nipagin in alcohol	9 g
Agar-Agar	64 g
Corn grits	220 g
Wheat germ	320 g
Brewer´s yeast	220 g
Ascorbic acid in water	25 g

Figure II.3: The oviposition sites for *Agrotis ipsilon* moths.

II.3.1.3. Rearing of Tomato leaf miner (*Tuta absoluta*)

Tuta absoluta could not be produced on artificial diets, and it was reared on tomato plants in a quarantine room. Tomato plants (Tomato Alissa F1, Nunhems Netherlands BV, Nunhem, Netherlands) were grown in a greenhouse at 22-28 °C and 16 h light: 8 h dark. The tomato seedlings were cultivated in plastic pots containing a suitable amount of growing substrate of 67% compost and 33% sand (v/v). *T. absoluta* rearing was established after obtaining *T. absoluta* eggs on tomato plants from Hochschule Geisenheim University, Geisenheim, Germany. The tomato plants infested with eggs were placed in Insect Rearing Tents (BugDorm-2120F, MegaView Science Education Services Co., Taiwan) (60 cm L * 60 cm W * 60 cm H) (Fig. II.4). Hatched larvae fed in mines on the tissue of tomato leaves. The larvae were regularly provided with fresh tomato plants. The larva developed to pupa through four larval instars. Pupation took place inside the mine, on leaves, on stems, on soil surface, or in soil. The adults emerged, mated, and the female moths started to lay eggs on fresh tomato plants, which were constantly added to the rearing cages. The rearing room was under controlled conditions at 25 °C and 16 h light: 8 h dark cycle.

Figure II.4: A plastic tent used for *Tuta absoluta* rearing.

II.3.2. Production of EPNs

The nematodes were produced *in vivo* using the last instar larvae of *G. mellonella* following a production protocol described by Kaya and Stock (1997). *G. mellonella* larvae were added to Petri dishes lined with two filter paper discs contaminated with infective juveniles (IJs) of a particular nematode isolate. After 48 hours, the dead larvae were transferred to so-called "White traps" described by Kaya and Stock (1997). The trap was introduced by White (1927). It consists of an inverted smaller Petri dish inside another bigger Petri dish. A piece of muslin was used to cover the inverted Petri dish, and its edges reach the bottom of the large Petri dish. Amount of Water (50 ml) was added to the Petri dish to moisten the piece of muslin and collect the emerged nematodes.

The dead larvae were placed on the wetted piece of muslin. The Petri dishes were covered with their lids and incubated at 25 °C (Fig. II.5). After 6-10 days, the new IJs migrated from the cadavers over the muslin and settled in the water. The nematodes were harvested by collecting the water from the Petri dishes. The collected EPNs suspension was cleaned to exclude the non-infective nematode stages and the dead host tissues. This was done by adding water to the nematode suspension, leaving it for about 10 min until the living nematodes settled down and then pouring out the excess water with any debris. The previous step was repeated twice. After that, the nematodes were stored in Petri-dishes at 12 °C.

Figure II.5: Dead *Galleria mellonella* larvae infected with *Steinernema carpocapsae* placed on White trap to collect nematode infective juveniles migrating through muslin piece into water in Petri dish.

II.3.3. Screening of EPNs efficacy against lepidopteran larvae in sand bioassays

Sand bioassays were conducted in which larvae of the target pests (*T. absoluta, A. ipsilon, S. littoralis,* and *H. armigera*) were exposed to 15 different EPN isolates (Table II.2). The bioassays were carried out in 24-well cell culture plates (Greiner bio-one GmbH, Frickenhausen, Germany). At first, sand was sieved through a sieve (2 mm) to remove big and other particles and sterilized at 60 °C for 24 h. Afterwards, the sand was distributed uniformly (1.5 gm/cell-well) into the wells of a plate to prepare the test vial. After settling of the sand, nematode IJs of a particular EPN isolate were applied in 300 μl water on sand per cell-well. The nematodes were applied at four doses with one, five, ten, or 20 IJs per cell-well. The correct number of IJs was picked up from a homogenous suspension of IJs in a Petri dish, using a micropipette and controlled by viewing through a stereomicroscope (Nikon SMZ745). Afterwards, larvae of one selected target pest were added singly to the cell-wells and were provided with a tomato leaf disc (7 mm diameter) (Fig. II.6). Only 20 out of 24 cell-wells per plate were occupied. Also, a control was included by preparing plates the same way except that only water without IJs was used. The plates were closed by their lids and incubated at 25 °C for 72 h in darkness. Subsequently, the number of living and dead larvae was recorded, and the larval mortality achieved by the particular EPN dose per larva was calculated. The target insect stage was the 3rd larval instar of the noctuid species and the 4th instar larvae of *T. absoluta*. Each nematode dose was applied on 40 larvae of each insect species, using two plates with 20 occupied cell-wells.

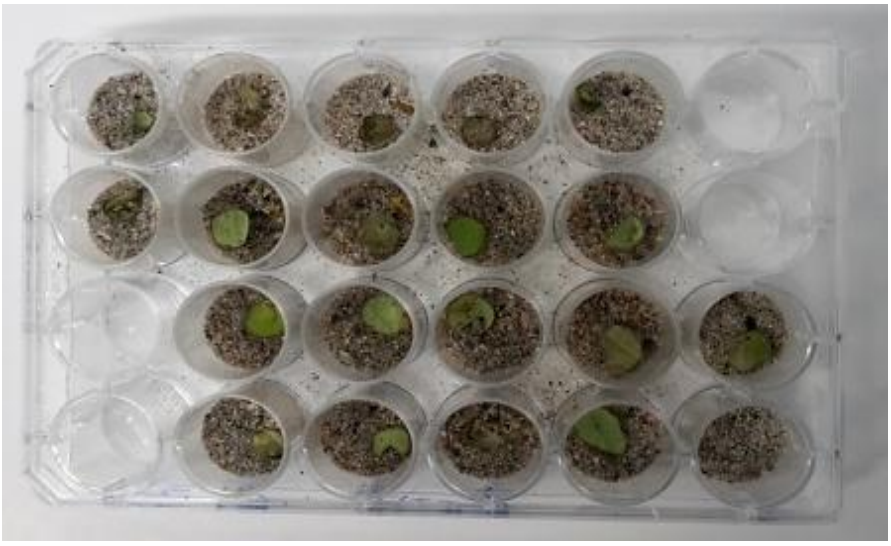


Figure II.6: 24-well cell culture plate used in Sand bioassay to test the efficacy of *Steinernema carpocapsae* (20 IJs/larva) on *Tuta absoluta* larvae.

Fifteen isolates from different regions were tested on the target insect species (Table II.2). The first eight isolates were introduced from the laboratory of Entomonematology, Pests and Plant Protection Department, National Research Centre, Giza, Egypt. These isolates were collected from different regions and maintained *in vivo* for more than seven years in the previously mentioned laboratory. The next two isolates were obtained from e-nema GmbH, Germany. The last five isolates were isolated at the beginning of this study in April 2015. The isolates were extracted from 15 soil samples collected from two different apple orchards located in the near of Rossdorf, Hesse, Germany. Each sample (750-1000 g soil) was taken using a sterilized hand shovel at a depth of 15-30 cm from two sides around apple trees. The soil samples were collected in plastic bags marked with each sample detail (the exact location and the collection date). Subsequently, the collected samples were kept at 15 °C in a cool box while transporting them to the laboratory. The samples were examined for containing EPNs following the insect-baiting method described by Bedding and Akhurst (1975). The soil was moistened by spraying water with a Hand-sprayer and was mixed carefully. Afterwards, the soil was divided and placed with 10 *G. mellonella* last instar larvae. The cups were covered by the lids. Small ventilation holes were made in the lids. The cups were incubated at 25 °C. After four days, the cups were checked, and the dead larvae were collected. The dead larvae were individually rinsed in water and placed in modified White traps (White, 1927). The nematode progeny (nematode IJs) that emerged after 10 days were collected and rinsed by adding water and let them settle down before removing the excess water. The isolated nematodes were tested for pathogenicity against *G. mellonella* last instar larvae to fulfill Koch's postulates (Kaya and Stock, 1997). The emerged nematode IJs were maintained *in vivo* in *G. mellonella* larvae.

All fifteen isolates were tested in bioassays on the lepidopteran target pests (*A. ipsilon, H. armigera, S. littoralis,* and *T. absoluta*) at different times. From these results, the four most promising EPN isolates were selected and subsequently tested on *B. tabaci* in leaf bioassay (Chapter III) and 4th instar larvae of *T. absolu*ta in sand bioassay and leaf bioassay (Chapter IV).

Table II.2: List of the nematode isolates used in laboratory screening against larvae of four lepidopteran insects.

	Nematode isolate	Code	Origin
1	*Steinernema carpocapsae*	BA2	South Sinai, Egypt
2	*S. carpocapsae*	S2	South Sinai, Egypt
3	*Steinernema glaseri*	Sg	New Jersey, USA
4	*Steinernema riobrave*	Sr	Texas, USA
5	*Steinernema feltiae*	Sf	Germany, e-nema GmbH
6	*Steinernema. abbasi*	abb	The Sultanate of Oman
7	*Heterorhabditis marelatus*	mar	Oregon, USA
8	*Heterorhabditis bacteriophora*	HP88	Utah, USA
9	*S. carpocapsae*	Scen	Germany, e-nema GmbH
10	*S. feltiae*	Sfen	Germany, e-nema GmbH
11	*Steinernema sp.*	J2	Rossdorf, Hessen, Germany
12	*Steinernema carpocapsae*	J7	Rossdorf, Hessen, Germany
13	*Heterorhabditis sp.*	J10	Rossdorf, Hessen, Germany
14	*Heterorhabditis sp.*	J12	Rossdorf, Hessen, Germany
15	*Heterorhabditis sp.*	J13	Rossdorf, Hessen, Germany

II.3.4. Data analysis

The objective of the screening was to identify those EPN strains, which are highly active against all lepidopteran target pests. Bioassays with the highly related lepidopteran larvae were considered replicates, irrespective of their species classification, to facilitate the identification process. Thus, four replicates (one based on *A. ipsilon*, one on *H. armigera*, one on *S. littoralis,* and one on *T. absoluta*) were obtained for EPN isolate and dosage. Each replicate consists of 40 larvae the number of dead and live tested larvae. The differences among the nematode isolates were analyzed within each dose separately. The statistical analysis and graphical presentation of results were performed using R software version "3.4.2" (R Core Team, 2017). Data of proportion were considered (the number of dead larvae versus the number of living larvae in each replicate) and consequently analysis of Deviance, assuming a general linear model (GLM) was applied. One GLM with binomial data distribution (Crawley, 2012) was fitted with EPN isolate as an explanatory variable and the number of present dead and living larvae as a response variable for each dosage. Model residuals were visually checked out, and dispersion was checked as well. The package

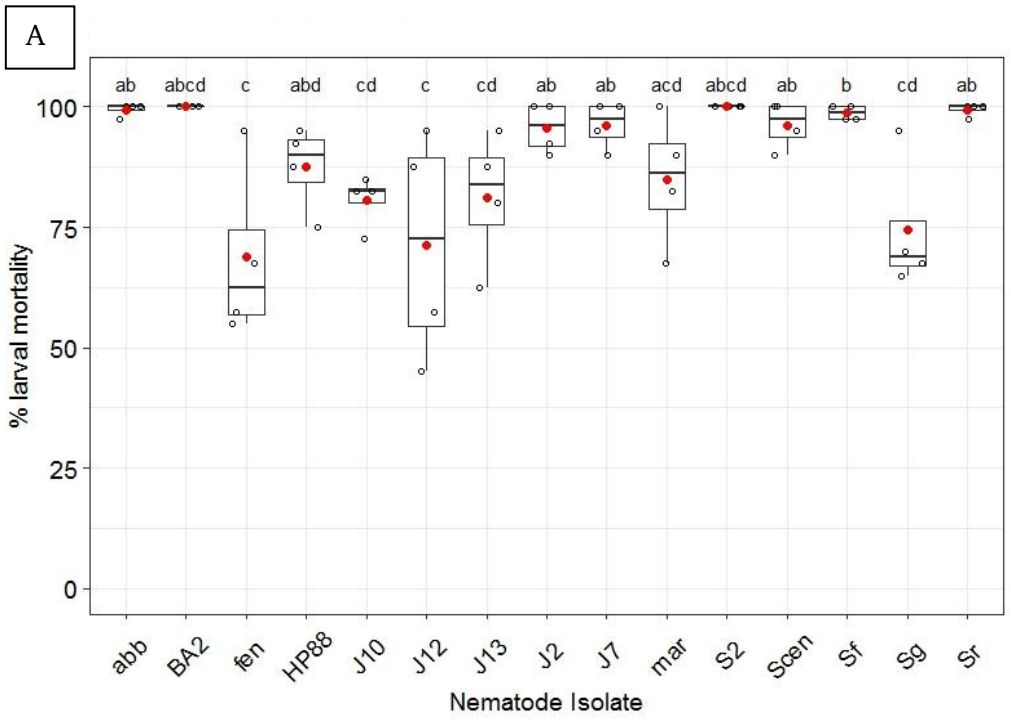
'emmeans' (Lenth, 2019) was used to calculate the estimated marginal means, posthoc tests at 0.05 significant level by the Tukey method, and 95% confidence intervals.

II.4. RESULTS

Bioassays against lepidopteran larvae

In all cases, the GLM fitted to the data revealed that mortality of lepidopteran larvae was affected by the EPN isolate used dosage: 20 IJs/larva: GLM: ~ isolate; $F=6.49$, $df=14$, $P < 0.0001$; 10 IJs/larva: GLM: ~ isolate; $F=4.36$, $df=14$, $P < 0.0001$; 5 IJs/larva: GLM: ~isolate; $F=4.56$, $df=14$, $P < 0.0001$; 1 IJs/larva: GLM: ~ isolate; $F=2.07$, $df=14$, $P = 0.03$. The larval mortality was due to the nematode present, as there was no mortality recorded in the control treatment. There were highly varying infectivity levels achieved by the screened EPN isolates within the same applied dose (Fig. II.7.A). The tested EPN isolates evoked at least 68% mortality in the tested insect larvae at the high dosage of 20 IJs/larva. At this dosage level, eight isolates caused above 90% larval mortality. They all belonged to *Steinernema* species (isolates abb, BA2, J2, J7, S2, Scen, Sf, and Sr). Seven isolates out of the previous ones infected more than 90% of the larvae at the dosage of 10 IJs/larva (Fig. II.7.B). They also achieved the highest larval mortality among the tested isolates when applied at a dose of 5 IJs/ml (Fig. II.7.C). Four isolates (abb, BA2, J7, and S2) killed at least 50% (median) of the treated larvae (Fig. II.7.D), even when applied with only one IJ per larva.

According to these results, four isolates were selected from the listed ones (Table II.1) to be considered in further evaluations of EPN isolates against the ETPC. From the three *S. carpocapsae* isolates, the most efficient one (*S. carpocapsae* BA2) was selected. Furthermore, *S. abbasi* abb, *S. feltiae* Sf, and *S. carpocapsae* J7 were chosen to consider a range of different species and origins.



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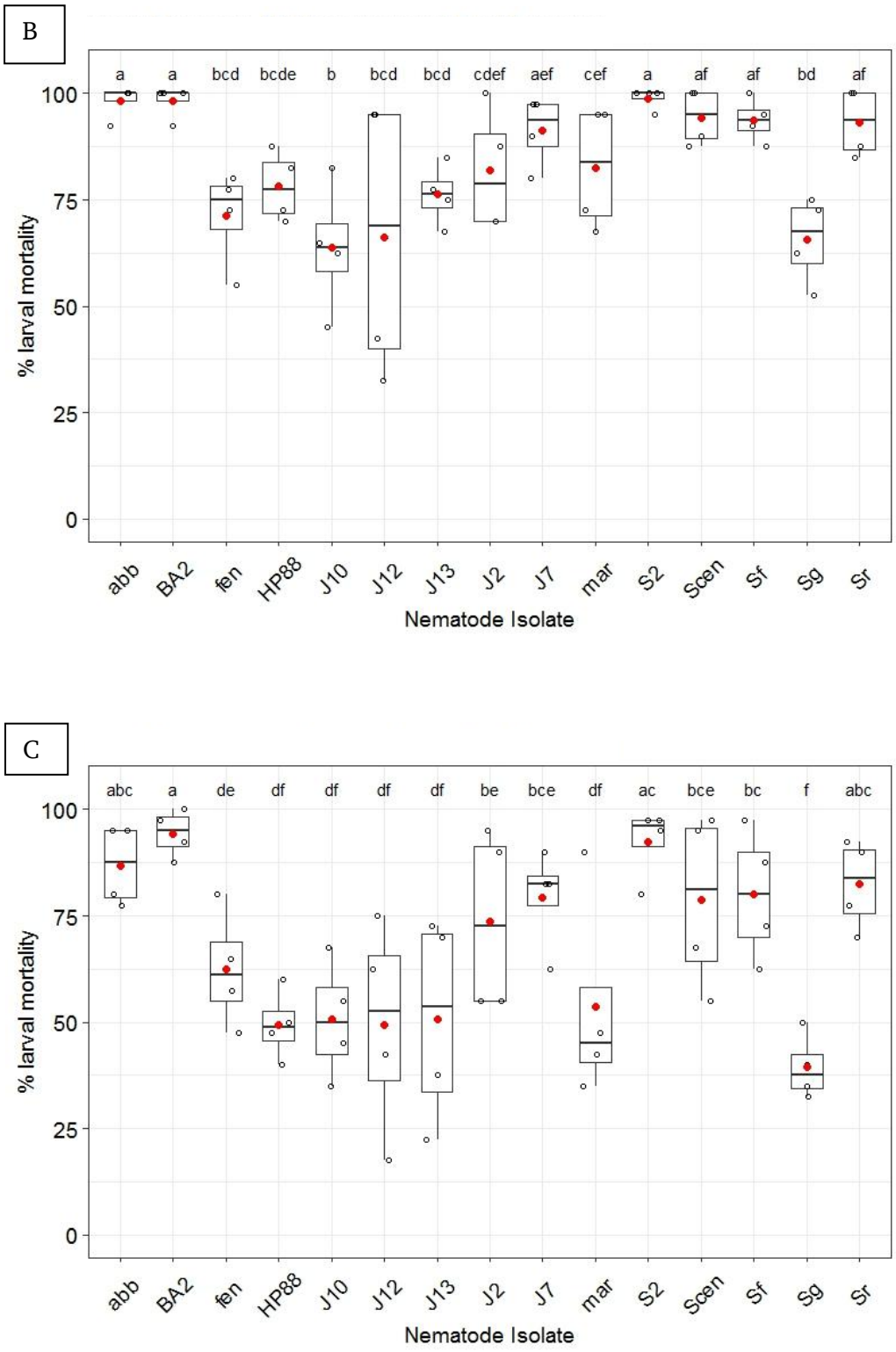

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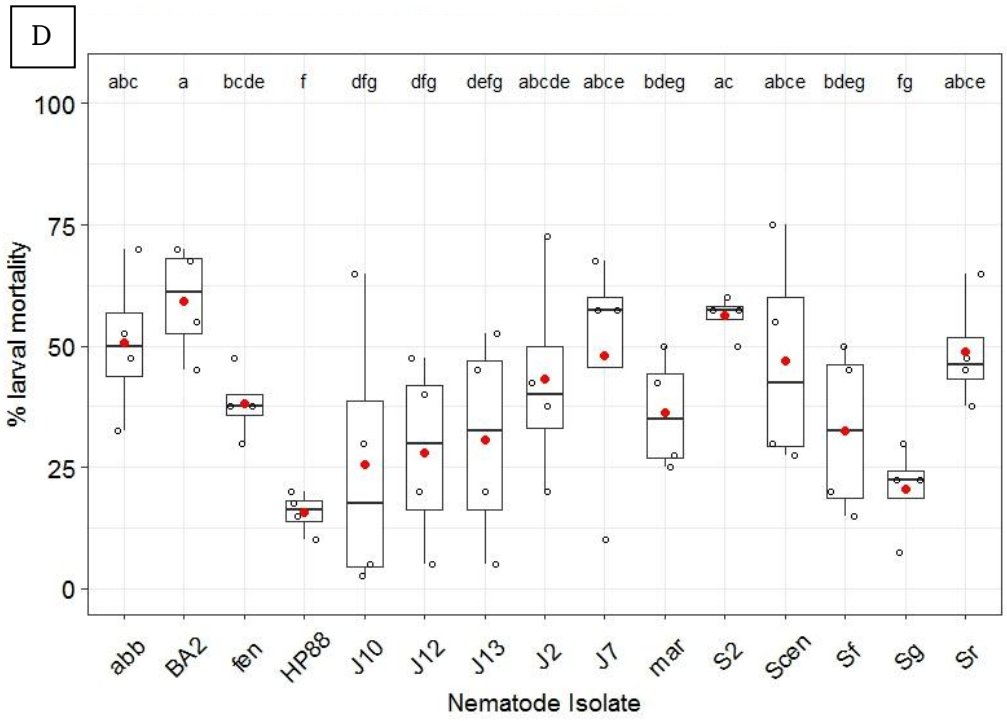

Figure II.7: Comparison of the efficacy of different nematode isolates (Table II.1) at different dosages (panel A: 20 IJs/larva; panel B: 10 IJs/larva; panel C: 5 IJs/larva; panel D: 1 IJ/larva) against larvae of four lepidopteran insect species (boxplot, n=4). Small black dots display replicates per isolate. The median is displayed by the horizontal line inside the boxplot. The estimated marginal mean is displayed by the red dot. The letters at the top indicate significant differences among the tested isolates according to Tukey test ($p < 0.05$).

II.5. DISCUSSION

Fifteen different EPN isolates, covering a range of species and origins, were evaluated against four lepidopteran pests attacking tomato crops in Egypt and other countries in this laboratory screening. Larvae of these species show a somewhat similar feeding pattern, as they attack leaves, stems, and fruits of tomato. Furthermore, these insects damage the plant over the entire growing season, and they often occur together. Therefore, this study aimed to find isolates with high virulence against all these target hosts, so that an equal optimal control can be achieved when applied in the field.

EPNs are known to exhibit different virulence and efficacy against potential host insects as a consequence of several internal and external factors. There are three features in some *Steinernema* nematodes that could explain their high virulence in comparison with *Heterorhabditis*. Firstly, most *Steinernema* nematodes use the ambusher strategy (sit and wait) to find a host. Moreover, some *Steinernema* nematodes showed the ability to use cruiser and ambusher strategies together, which increases their chances to find the host. Secondly, they have nictation behavior in which IJ stands on its posterior part and moves its body in all directions, searching for a host (Campbell and Gaugler, 1993). Thirdly, they have the ability to make a loop while standing and then jumping, which could help it reach and attach to a host (Campos-Herrera, 2015). All the previous characteristics could enhance the capability of *Steinernema* nematodes to reach, attach to, and infect their hosts. Furthermore, all the previous three features have not been recognized in the nematodes of *Heterorhabditis* genus

(Lewis et al., 2006). On the other hand, IJs of *Heterorhabditis* nematodes have a dorsal tooth that could be used to penetrate the insect cuticle between the segments (Griffin et al., 2005). Different efficacy levels of EPNs were reported by many authors (Sharma et al., 2011, and Biondi et al., 2018). This emphasizes the need for screening and selecting the most efficient EPN isolate against the target insect pest.

The EPN isolates screened in this experiment were from different species and isolates from different origins. The results reported by Seenivasan and Sivakumar (2014) showed virulence differences among the strains within the nematode species. The virulence of 27 EPN strains was evaluated against the cotton leafworm *Spodoptera litura* (Fabricius), the spotted bollworm *Earias vittella* (Fabricius), and *H. armigera*. The tested strains consisted of 16 *S. carpocapsae*, one *S. monticolum* (Stock, Choo, & Kaya), three *Steinernema siamkayai* (Stock, Somsook & Reid), and seven *H. bacteriophora*. The virulence of the applied strains differed significantly against the target insects' larvae. *H. bacteriophora* and *S. carpocapsae* were more virulent than *S. monticolum* and *S. siamkayai* on *S. litura*, *E. vittella*, and *H. armigera*. Nevertheless, the virulence rates differed among the seven *H. bacteriophora* strains and 16 *S. carpocapsae* strains. The larval mortalities achieved by the strains of *S. carpocapsae* were 38.9-88.9% of *S. litura*, 31.5-92.7% of *E. vittella*, and 42.4-87.8% of *H. armigera*. Similar variations were detected among the strains of *H. bacteriophora*. The reported results suggested that virulence differs among the strains and not unique within a particular species. The authors found that the invaded IJ numbers of the more virulent EPN strains were significantly more in comparison with the less virulent strains. Therefore, evaluating the efficacy of different isolates within one species was important.

The general finding that all four target host species were susceptible to EPN infection confirmed previously published results. Fetoh et al. (2009) showed that *A. ipsilon* larvae were successfully infected by both species *S. carpocapsae* and *H. bacteriophora*. *Heterorhabditis* isolates killed all larvae of *A. ipsilon* when applied at 100 IJs/larva (Shamseldean et al., 2009). In the early study, El Kifl (1980) found that all larval stages of *S. littoralis* were successfully infected by *Neoaplectana carpocapsae* (=*S. carpocapsae*) in leaf bioassays on castor leaves, whereas Abdel-Kawy (1985) stated that the 3rd and 4th instar of *S. littoralis* larvae were the most susceptible ones. Salem et al. (2007) reported that the nematodes of *S. carpocapsae* S2 and *S. carpocapsae* All were the most efficient isolates among the tested nematode isolates against *S. littoralis* using cabbage leaf disks bioassay. Shamseldean et al. (2009) reported that *Heterorhabditis* isolate obtained 100% larval mortality in *S. littoralis* when applied at a dose of 250 IJs/larva in the laboratory.

In case of *H. armigera,* Tahir et al. (1995) reported that *S. carpocapsae*, *Heterorhabditis* sp., and *Steinernema riobrave* were pathogenic to *H. armigera* larvae. Many studies have also confirmed the pathogenicity potential of EPNs on *T. absoluta* larvae under laboratory, greenhouse, or open field conditions. Larvae were susceptible to *S. carpocapsae*, *S. feltiae*, and *H. bacteriophora* under laboratory controlled conditions as reported by Batalla-Carrera et al. (2010). They applied the nematodes at a dose of 50 IJs/cm^2 on Petri dishes. These applications resulted in larval mortality ranged between 86.6% and 100%. Also, Garcia-del-Pino et al. (2013) reported that EPNs application on soil against the larvae that go into the ground to pupate caused high mortality rates under controlled laboratory conditions. Furthermore, Van Damme et al. (2016) reported high mortality in *T. absoluta* larvae inside

their mines after applying *S. carpocapsae* and *S. feltiae* on tomato leaf disks under laboratory conditions.

Nematode isolates, which showed high virulence against all target hosts, all belong to the genus *Steinernema*: *S. carpocapsae*, *S. abbasi*, *S. riobrave*, and *S. feltiae*. Similarly, Abonaem (2013) also found that *S. carpocapsae* was more effective than *H. bacteriophora* on either *S. littoralis* or *Agrotis ipsilon* 4th instar larvae on corn plants in the open field. Here, *S. carpocapsae* infected all larvae of *S. littoralis* and *A. ipsilon,* whereas *H. bacteriophora* killed only 33.57% of *S. littoralis* larvae and 83.81% of *A. ipsilon* larvae. *Steinernema* species and isolates were also more effective than *Heterorhabditis* against *T. absoluta* (Batalla-Carrera et al., 2010). At a dose rate of 25 IJs/cm^2, the mortality in *T. absoluta* larvae was 100% with *S. feltiae*, 85.7% with *S. carpocapsae,* and 78.6% with *H. bacteriophora*. In another study, nematodes of *S. feltiae* and *S. carpocapsae* caused similar mortalities in *T. absoluta* larvae at the applied inoculation rates while the efficacy of *H. bacteriophora* was lower (Türköz and Kaskavalci, 2016). Also, Van Damme et al. (2016) obtained better results with *S. carpocapsae* and *S. feltiae* than with *H. bacteriophora* against the same host insect.

Effects found for the included five *Heterorhabditis* isolates were comparatively lower than for *Steinernema* isolates at all tested dosages. *Heterorhabditis* is probably less effective at such low dose rates as applied in the current study against lepidopteran pests (maximum 20 IJs/larva). Shamseldean et al. (2009) found similar results when they tested three *Heterorhabditis* isolates against *S. littoralis* and *A. ipsilon* larvae in the laboratory. They applied the nematodes at doses of 5, 50, 100, 250, 500, and 1000 IJs/larva. Isolates of *Heterorhabditis* caused larval mortality of 100% only at doses of 100 IJ (against *A. ipsilon*), or 250 IJ/larva (*S. littoralis*) or higher. But one report stated that the efficacy of *H. bacteriophora* was relatively higher than *S. riobrave* against *S. littoralis* larvae under laboratory conditions (86% and 71% mortality, respectively) (Shairra and Noah, 2014). One possible explanation of the low efficacy of *Heterorhabditis* nematodes, that their IJs are cruisers. The applied IJs may move deeply in the sand column and do not meet the target larva on the soil surface. In the bioassay, the nematodes were added to the sand before adding larva. By bringing it all together, when the nematodes are applied at a low dose rate, IJs enter into the soil, and there are no sufficient IJs to infect larva on the soil surface.

Comparable levels of high efficacy were found for *S. feltiae* and *S. riobrave* within the different dosages at the present study. The commercial availability or suitability for mass production was taken into consideration to select one of them for further experimentation. In contrast to S. *riobrave*, isolates of *S. feltiae* are available from several companies worldwide (e.g. e-nema GmbH, Schwentinental, Germany), and even the selected isolate *S .feltiae* Sf, is produced commercially. Although *S. riobravae* has already been tested in several studies against many pests, it has not yet been widely produced for commercial use. Furthermore, this species is only known from North America and would therefore be more difficult to consider for field application in many countries as a non-native species. Therefore, the nematode species of *S. feltiae* was selected instead of *S. riobrave*.

Two isolates (BA2 and J7) of the species *S. carpocapsae* were selected, mainly due to the high efficacy demonstrated in this screening. Besides this, BA2 was collected in Sinai region in Egypt, therefore promising good adaptation to environmental conditions in Egypt as a native

organism of this target country. On the other hand, isolate *S. carpocapsae* J7 was found in Germany during the present work. It achieved high mortality even at the lowest dose and may offer other capabilities not yet known, as this candidate was not studied before. In addition, also S. *abbasi* performed well in the conducted screening and was chosen due to its origin from the Sultanate of Oman with semi-arid tropic conditions which are comparable to the Egyptian environment.

The objective of the screening study was to establish a feasible "shortlist" of potential candidate EPN isolates for control of the ETPC. These candidates were subject to more detailed studies in the following against *B. tabaci* (Chapter III) and *T. absoluta* (Chapter IV till Chapter VI).

Chapter III: Efficacy of selected entomopathogenic nematodes to control the Whitefly *Bemisia tabaci* under laboratory conditions

III.1. ABSTRACT

The Whitefly *Bemisia tabaci* is one of the most destructive insect pests on tomato crops in Egypt. Four nematode isolates found to be efficient on lepidopteran pests in a previous study were tested against the whitefly to create a unique nematode-based control system against the Egyptian tomato insect pest complex (ETPC). The isolates *Steinernema carpocapsae* BA2, *Steinernema feltiae* Sf, *Steinernema abbasi* abb, and *S. carpocapsae* J7 were applied at three concentrations (1000, 2000, and 4000 IJs/ml) in 0.3% Tween 80 suspensions on tomato leaves which had been infested with 2nd nymphal instars of the whitefly before. All tested isolates were able to infect the target host stage successfully. The nematodes of *S. feltiae* were significantly the most efficient within the concentrations of 2000 and 4000 IJs/ml. The nymphal mortality obtained by *S. carpocapsae* BA2 was not significantly different from that obtained by *S. feltiae* Sf within the concentration of 1000 IJs/ml.

III.2. INTRODUCTION

The tobacco whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae), is a highly polyphagous pest that attacks many important crops such as cucumber, sweet potatoes, cotton, potatoes, and tomatoes. It is a serious pest in almost all the countries around the world. In Egypt, *B. tabaci* is a major pest on tomatoes cultivated either under greenhouses or in open fields. It attacks the crop during all growth stages until harvest. This insect causes huge losses to the crop between April and November (ElGindy, 1997).

The whitefly life cycle starts when the female flies lay eggs on the underside of the leaves. Eggs hatch to the first nymphal instars, which are crawlers and are the only mobile nymphal instar. It moves to find a suitable feeding site and molt to the second nymphal instar, which is immobile and sessile on the underside of the leaf. Each instar of the first three nymphal instars develops within 2-4 days, depending on the environmental conditions. The fourth nymphal instar is called pupa, which develops within 6-7 days to the adult fly.

B. tabaci causes direct damages to the crop by feeding and indirect damages by transmitting plant pathogenic viruses (Brown, 2010). The nymphs and the adults feed on the lower surface of the leaf, inserting their mouthparts and sucking the plant sap. Their feeding causes yellow spots on the upper surface of the leaf, which further extend during the infestation. All feeding stages produce honeydew that covers the leaf surface. It can impede photosynthesis and induces sooty mold fungi growth on the leaves and the fruits as well (Stansly and Natwick, 2010). Furthermore, *B. tabaci* is a vector for some plant pathogen viruses that cause significant losses in the infested crops. The most common viruses are Tomato chlorosis virus (TCV) and Tomato yellow leaf curl virus (TYLCV). These viruses cause some symptoms such as Leaf curling, mosaics, or yellowing (Czosnek and Laterrot, 1997; Gorovits et al., 2013).

Growers use chemical insecticides to control or eradicate this serious pest. As a consequence of extensive use of chemical insecticides, *B. tabaci* developed resistance to nearly all groups of insecticides (Ahmad et al., 2002; Wang et al., 2010) and even novel active ingredients quickly lose their effectiveness against this pest (Dennehy et al., 2010). For this reason, other control methods based on biological control agents need to be developed and integrated into pest management programs against *B. tabaci*. Parasitoids, predatory mites (Cuthbertson, 2014), and entomopathogenic agents such as nematodes (Cuthbertson et al., 2003, 2007a,b) and fungi (Cuthbertson and Walters, 2005; Cuthbertson et al., 2005, 2012;) were shown to be possible tools in *B. tabaci* control. The laboratory experiments described in this chapter aimed to prove the ability of particular EPN isolates to infect the whitefly *B. tabaci*. The selected isolates had been shown to be highly virulent for different lepidopteran pest species attacking tomato crops in Egypt, such as *Helicoverpa armigera, Spodoptera littoralis, Agrotis ipsilon,* and *Tuta absoluta* (Chapter II). Due to their broad host spectrum, EPNs are particularly interesting when it comes to developing a biological control strategy not only against the whitefly but also against as many other important pests on tomatoes as possible. The whitefly is a significant member of the so-called Egyptian Tomato insect Pest Complex (ETPC). However, life history, susceptible stages, and especially feeding and damaging pattern of the whitefly significantly differs from that of lepidopteran members of the ETPC. All stages of the whitefly occur only on leaves; they feed by sucking the sap and they are very small in size. Rapid development of multiple generations takes place, so that also vulnerable stages are available only for short times, but occur frequently. All insect stages could occur at the same time on the plant or even on the same leaf.

The general approach of the investigation focuses on the identification of suitable EPN isolates which can successfully tackle the whole ETPC. Sand bioassay is a perfect and standardized test procedure to check the effectiveness of EPN isolates with different characteristics and behaviors on larval stages of various insect orders (Glazer and Lewis, 2000; Grunder et al., 2005), but they are unsuitable for testing EPNs against phloem sap feeders like whiteflies. This made the screening of EPNs on the whole ETPC in one bioassay inappropriate and EPN isolates selected from primary screening against lepidopteran pests were tested for efficacy on immobile 2nd nymphal instar on tomato leaves.

III.3. MATERIALS AND METHODS

III.3.1. Rearing of the Whitefly (*Bemisia tabaci*)

B. tabaci nymphs were obtained from Bayer AG, Leverkusen, Germany. The insects were reared on tomato plants in Insect Rearing Tents (BugDorm-2120F) (60 cm L * 60 cm W * 60 cm H) (MegaView Science Education Services Co., Taiwan) at 25 °C, 50-70% RH, and 16 h light: 8 h dark cycle (Fig. III.1). New tomato plants were added to the rearing cages every week to maintain the culture. The 2nd instar nymphs were used on the bioassays to assess the nematode efficacy.

Figure III.1: Insect rearing tents used for rearing the whitefly *Bemisia tabaci* on tomato plants.

III.3.2. Infestation of tomato plants

Specific infestation of host plants was performed by distributing about 100 adults of *B. tabaci* on five tomato plants (six leaves old) in an Insect Rearing Tents (BugDorm-2120F) (60 cm L *60 cm W *60 cm H) to allow a period of 48 h for oviposition (Fig. III.1). Tents were placed in a controlled rearing chamber at 25 °C, 50-70% RH, and 16 h light: 8 h dark cycle. After exposure, the plants were cleaned from adult whiteflies, moved to new tents, and kept for further 12 days at the same conditions till the emergence of the 2nd nymphal instars. Then, infested leaflets were collected and used in the bioassay.

III.3.3. Set up of the bioassay

Tomato leaflets infested with at least five *B. tabaci* 2nd nymphal instar were collected to be treated with nematode suspensions (Fig. III.2). The EPN isolates *S. carpocapsae* BA2 & J7, *S. feltiae* Sf, and *S. abbasi* abb were applied at three concentrations (1000, 2000, and 4000 IJs/ml) in water suspensions, containing 0.3% Tween 80 as an adjuvant. Each concentration was prepared in a volume of 250 ml before the application. Of this, a volume of 50 ml of the nematode suspension was put into a hand sprayer (50 ml) and sprayed on 10 tomato leaflets one after the other until runoff. Immediately after the application, each leaflet was placed in a plastic box (15 cm L * 10 cm W * 5 cm H). The leaflets were placed with the underside facing up. Leaflet petioles were stuck into moistened peat moss to keep them fresh. Thereafter, the boxes were covered by lids and incubated at 25 °C, 16 h light: 8 h dark cycle. The previous application was performed four times on different days and, in total, 40 leaflets were obtained for each concentration of each nematode isolate. Other 40 leaflets, serving as control, received only 0.3% Tween 80 without nematodes. After 72 h incubation, the leaflets were checked under a binocular microscope, and dead and living nymphs were counted.

III.3.4. Data analysis

The objective of the bioassay was to investigate the efficacy of four nematode isolates against *B. tabaci* 2nd instar nymphs. The comparison among isolates and different concentrations was assessed. Each concentration had four replicates. Each replicate consisted of the number of dead and living nymphs on ten tomato leaflets, which had been treated at the same time by the nematode suspension. The statistical analysis and graphical presentation of results were performed using R software version "3.4.2" (R Core Team, 2017). Numbers of dead and living *B. tabaci* 2nd instar nymphs in response to different EPN isolates and applied concentrations were compared to estimate the efficacy of these EPN isolates. Data of proportion were considered (number of dead nymphs versus the number of living nymphs in each replicate) and consequently analysis of Deviance, assuming a general linear model (GLM) was applied. One GLM with binomial data distribution was fitted with EPN isolate, concentration, and the two-way interaction between isolate and concentration as explanatory variables and the number of present dead and living nymphs as a response variable. Model residuals were visually checked out, and dispersion was checked as well. The package 'emmeans' (Lenth, 2019) was used to calculate the estimated marginal means, posthoc tests at 0.05 significant level by the Tukey method, and 95% confidence intervals.

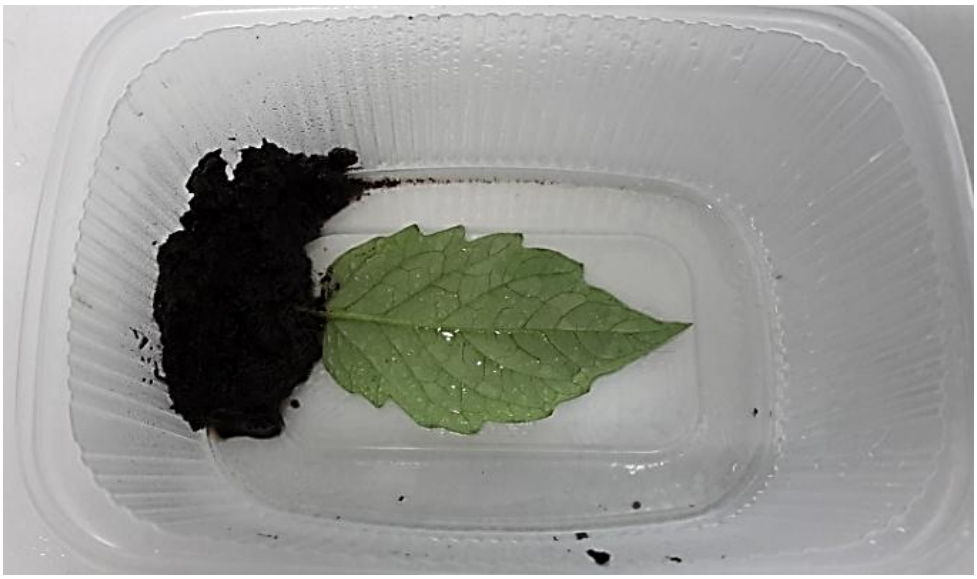

Figure III.2: Plastic box containing a tomato leaflet infested with *Bemisia tabaci* 2nd nymphal instar and treated with *Steinernema carpocapsae* BA2.

III.4. RESULTS

All the tested isolates successfully infected the whitefly nymphs (Fig. III.3). No nymphs died in the control treatment, and statistical comparison was restricted to EPNs treatments. Thereby, nymphal mortality was affected by isolate, concentration, and the interaction of these explanatory variables (GLM: Probit (cbind (dead, living)) ~ Isolate * Concentration: LRT=887.36, df=12, $p<0.001$). The isolate *S. feltiae* Sf consistently achieved the highest mortality (80%) in *B. tabaci* nymphs at each concentration level (Fig. III.4), although not

significantly so compared to *S. carpocapsae* BA2 at lower concentrations of 1000 or 2000 IJ/ml. Application of *S. feltiae* at a concentration of 4000 IJ/ml caused 80% mortality in *B. tabaci* nymphs and was significantly higher compared to the other isolates and concentrations (Fig. III.4). The isolates *S. abbasi* abb and *S. carpocapsae* BA2 or J7 elicited similar effects (but less than 50% mortality) when applied at 4000 IJs/ml. At the lowest concentration of 1000 IJs/ml, BA2 was similarly effective to *S.feltiae*, but mortality was generally low.

Figure III.3: Whitefly nymphs (A) alive, (B) and (C) infected by *Steinernema feltiae*. The length of whitefly 2nd nymphal instar=0.29-0.38 mm.

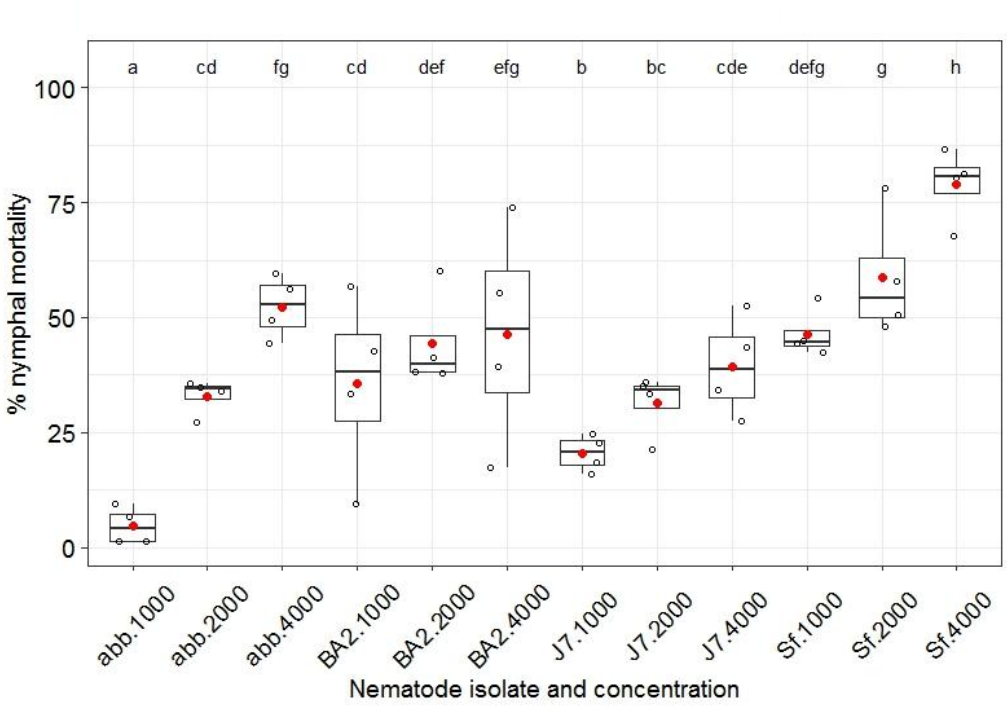

Figure III.4: Comparison among four EPN isolates *Steinernema abbasi* abb (abb), *S. carpocapsae* BA2 (BA2) and J7 (J7), and *S. feltiae* Sf (Sf) applied at three different concentrations (1000 IJs/ml, 2000 IJs/ml, and 4000 IJs/ml), and their effect on the mortality of the whitefly, *Bemisia tabaci* 2nd nymphal stages in leaf bioassay. Boxplots are shown with individual observed values (n=4) as jittered points. The median is displayed by the horizontal line inside the boxplot. The estimated marginal means are displayed by the red dots estimated from the fitted model. The letters at the top indicate significant differences among the tested treatments (Isolates and concentrations) according to posthoc tests ($p < 0.05$).

III.5. DISCUSSION

The objective of these experiments was to determine if EPN isolates, which have been shown to be effective against ETPC lepidopteran species in a previous screening, could cause sufficient mortality of whitefly nymphs. Appropriate leaf bioassays were used to evaluate the nematode efficacy, but also to ensure whitefly survival in the control treatment. The target stage was the 2nd nymphal instar because this stage was the most susceptible to nematode infection according to other publications (e.g. Cuthbertson et al., 2003; Cuthbertson et al., 2007a,b). Bioassays were conducted two weeks after oviposition and incubation period of infested plants, because then 2nd nymphal instars occur (Cuthbertson et al., 2007b).

According to this study, *S. feltiae* Sf proved to be the most effective of four test isolates against *B. tabaci* 2nd nymphal instars. Also, Cuthbertson et al. (2003) investigated *S. feltiae* activity against the four nymphal instars of *B. tabaci* on tomato and verbena. They applied the nematode at the high concentration of 10,000 IJs/ml, formulated in 0.02% Agral (non-ionic wetting agent), at 20 °C and found that the second nymphal instar was the most susceptible larval stage. They concluded that *S. feltiae* applications can reduce about 65% of *B. tabaci* 2nd nymphal instars on both tomato and verbena foliage. In another study, the efficacy of *S. feltiae* and *H. bacteriophora* was evaluated against the greenhouse whitefly *Trialeurodes vaporariorum* on cucumber and pepper (Rezaei et al., 2015). The susceptibility of adults and second nymphal instars to the two nematode species was tested at different rates (0, 25, 50, 100, 150, 200, and 250 IJs/cm^2) under laboratory conditions. Afterwards, experiments against 2nd nymphal instars were conducted on pepper and cucumber plants under greenhouse conditions. The adjuvant Triton X-100 (0.1%) was added to the nematode suspension for enhancing nematode activity. Both nematode species were able to infect both adults and nymphs, but, according to LC_{50} values, *S. feltiae* was more effective than *H. bacteriophora* in the laboratory bioassays. Furthermore, *S. feltiae* achieved the same nymphal mortality as *H. bacteriophora* in the greenhouse experiments, but already at lower concentrations. Application of *S. feltiae* at a dosage of 250 IJs/cm^2 gained the highest mortality in *T. vaporariorum* 2nd nymphal instars (49±1.23%) on cucumber under greenhouse conditions. In general, the efficacy of EPNs against *T. vaporariorum* on cucumber was significantly higher than on pepper, indicating that also the host plant of the whitefly may play an important role.

Whiteflies are difficult targets for EPNs compared to lepidopteran pests, because even high concentrations of IJs result in relatively low mortalities, at least under greenhouse or semi-field conditions. Nevertheless, mortality rates of up to 80% were observed in the current leaf bioassays by application of the EPN isolate *S. feltiae* Sf. The other EPN species also achieved mortalities above 50% when applied at high concentrations. These results proved that EPN isolates that are effective against lepidopterans of the ETPC can also successfully attack whiteflies. Clearly, whiteflies were found to be less susceptible to infection by EPNs than lepidopteran hosts, but this claim needs to be proven in further experiments.

Chapter IV: Screening of entomopathogenic nematodes against the tomato leaf miner, *Tuta absoluta*, an invasive tomato pest in Egypt.

IV.1. ABSTRACT

Since its introduction in 2009, tomato leafminer, *Tuta absoluta* has become the most dangerous pest on tomatoes in Egypt. Therefore, it was selected as the key target among the Egyptian tomato insect pest complex (ETPC) to develop a biocontrol system based on Entomopathogenic nematodes (EPNs). Four EPN isolates (*Steinernema carpocapsae* BA2, *S. feltiae* Sf, *S. abbasi* abb, and *S. carpocapsae* J7) were tested against *T. absoluta* larvae in sand and leaf bioassays under laboratory conditions. These EPNs were found to be the most efficient among 15 isolates in a previous screening against four lepidopteran species (Chapter II). All isolates successfully infected *T. absoluta* larvae when exposed directly in the sand bioassay. There were limited differences among the isolates at the doses applied. All isolates were capable of attacking *T. absoluta* larvae on tomato leaves inside and outside the mines, as shown by the leaf bioassay. The calculated LC_{50} values ranged between 112 IJs/ml (*S. feltiae* Sf), 103 IJs/ml (*S. carpocapsae* J7), 82 IJs/ml (*S. abbasi* abb), and 44 IJs/ml (*S. carpocapsae* BA2), suggesting that *S. carpocapsae* BA2 is the best candidate for Tomato leafminer control.

IV.2. INTRODUCTION

Tomato is the most cultivated vegetable crop in Egypt and it is a target for continuous pest infestation from the first day in the field to the last day of harvest. Nowadays, the invasive tomato leafminer moth, *Tuta absoluta* (Meyrick) (Lepidoptera, Gelechiidae) is the most destructive insect pest on tomatoes in Egypt. This insect was introduced to Egypt in 2009, coming from Spain through Libya (Khidr et al., 2013; Ata and Megahed 2014; Goda et al., 2015; Darbain et al., 2016). Since then, it has displaced the whitefly *Bemisia tabaci* (Gennadius) from the top of most important insect pests on tomatoes. The seriousness of this pest is caused by the short generation time (on average one generation per month), followed by the establishment of many generations per year, leading to pest outbreaks and permanent impact on the crop. Furthermore, the larvae attack the plant leaves, stems, and fruits, resulting in an immense loss in the yield quantity and quality. *T. absoluta* infestation can cause up to 80-100% losses if the pest is not controlled (Desneux et al., 2010). Standard control methods against this pest are currently based on chemical insecticides, so it is important to introduce new alternative methods based on biocontrol agents. Some alternative approaches, using beneficial insects such as *Trichogramma* sp. (Schäfer and Herz, 2020) or insect viruses (Ben Tiba et al., 2019), are promising, but are not available everywhere and - especially in the case of microbials - require registration as plant protection products. On the other hand, the use of entomopathogenic nematodes (EPNs) as antagonists could be an excellent option. They have some advantages such as their ability to kill their hosts quickly within only 48 h, they can control several host species simultaneously and they can be easily applied using the usual application tools, also on a larger scale. Besides that, EPNs can be produced *in vivo* and *in vitro*, and they do not cause any hazard to vertebrates or plants. EPNs have been used against many insect pests on different crop systems (Grewal et al., 2005). The

majority of applications take place against soil-dwelling pests, but there are also some examples of uses against target pests above ground or on foliage such as Diamondback moth, *Plutella xyllostella* Linnaeus (Lepidoptera: Plutellidae) (Schroer et al., 2005; Sáenz et al., 2020). Because *T. absoluta* larvae feed on the foliage, the foliar application is demanded. There already exist some studies on EPNs efficacy against *T. absoluta* larvae on tomato leaves. Batalla-Carrera et al. (2010) conducted a leaf bioassay under laboratory conditions and recorded high infection levels in *T. absoluta* larvae inside their galleries. Also, the applications of *S. feltiae*, *S. carpocapsae,* and *Heterorhabditis bacteriophora* resulted in high mortality rates in *T. absoluta* larvae inside their mines on tomato leaf disks under laboratory conditions (Van Damme et al., 2016). Moreover, Türköz and Kaskavalci (2016) tested *S. feltiae* against *T. absoluta* larvae inside the mines but these trials resulted in low mortality.

T. absoluta is now a big problem in Egyptian tomato production and can be the dominant species of the Egyptian Tomato insect Pest Complex (ETPC). Those EPNs that have shown promising activity against lepidopteran pests of the ETPC (Chapter II) were assayed against this insect as well. General activity against *T. absoluta* was explored in the standard sand bioassay. Then, it was tested whether the EPNs were able to reach *T. absoluta* larvae on leaves by entering mines and infecting larvae therein.

IV.3. MATERIALS AND METHODS

IV.3.1. Rearing of Tomato leaf miner (*Tuta absoluta*)

Tomato plants (Tomato Alissa F1, Nunhems Netherlands BV, Nunhem, Netherlands) were grown in a greenhouse at 22-28 °C and 16 h light: 8 h dark cycle. The tomato seedlings were cultivated in plastic pots containing a suitable amount of growing substrate of 67% compost and 33% sand (v/v). Rearing of *T. absoluta* was performed on tomato plants in insect Rearing Tents (BugDorm-2120F) (60 cm L * 60 cm W * 60 cm H) (MegaView Science Education Services Co., Taiwan). The rearing room was under controlled conditions at 25 °C and 16 h light: 8 h dark cycle.

IV.3.2. Production of Entomopathogenic nematodes

EPN isolates of *S. carpocapsae* BA2 (Hussein and Abou El-Souud 2006), *S. feltiae* Sf, *S. abbasi* (Elawad et al., 1997, isolate abb), and *S. carpocapsae* J7 were used in this study. These isolates had been selected as the most efficient isolates among 15 isolates against the lepidopteran tomato pests *Agrotis ipsilon*, *Spodoptera littoralis*, *Helicoverpa armigera*, and *Tuta absoluta* (Chapter II).

The first three isolates were obtained from the collection of the Insect nematology group, Pests and Plant Protection Department, National Research Centre, Giza, Egypt. The fourth isolate, *Steinernema carpocapsae* J7, was isolated from soil samples collected from an apple orchard in Rossdorf, South-Hessia, Germany in the year 2015. The isolate was identified as *Steinernema* morphologically and molecular identification, based on the ITS2-marker, suggests that it is a *S. carpocapsae* species (Ruoff, JKI, Darmstadt working group virology, unpublished). The nematode isolates were maintained as *in vivo* cultures in *Galleria mellonella*

last instar larvae at 25 °C (Chapter II). Infective juveniles (IJs) were collected within three days of emergence from host cadavers and stored in water at 12 °C and darkness for not more than 10 days until using.

IV.3.3. Sand-Bioassay

The sand bioassay was conducted to prove any differentiation among the four top-ranked EPN isolates from the previous screening in efficacy versus *T. absoluta* larvae. The nematodes were applied at four doses (1, 5, 10, or 20 IJs/larva/cell-well) against *T. absoluta* 4th instar larvae. Each dose was applied on 40 separate larvae in two 24-Cell-Well plates. The method was as described in the previous bioassay (Chapter II). Also, a control was included by preparing plates the same way, except that only water without IJs was used. In total, the bioassay was independently replicated three times at different time points.

IV.3.4. Leaf-Bioassay

It was necessary to obtain larvae of a particular instar within mines on tomato leaves for the leaf bioassay. About 100 newly emerged *T. absoluta* moths were placed together with ten tomato plants (six leaves old) in a tent (60 cm L * 60 cm W * 60 cm H) to allow oviposition at 25±1 °C, 40-70% RH, and 16 h light: 8 h dark cycle. Plants with eggs were transferred into a clean plastic cage 48 h later, supplied regularly with water, and maintained under the same temperature and light conditions. Eggs hatched, and the larvae developed within two weeks into the fourth instar, ready for use in the trials. Then, infested leaflets were collected (Fig. IV.1). They contained, on average, three 4th instar larvae in their mines.

Figure IV.1: Tomato leaflets infested with *Tuta absoluta* larvae to be used for the bioassay.

EPNs were applied as IJs at seven concentrations: 15, 30, 60, 125, 250, 500, and 1000 IJs/ml in water. The nematode suspension of each concentration was prepared in 300 ml

water before the applications. Afterwards, a volume of 50 ml from the prepared suspension was sprayed on 10 tomato leaflets using a hand sprayer (volume: 50 ml). The application was performed by holding a single leaflet in one hand and spraying the nematode suspension on both sides of the leaflet till runoff. The application was done four times for each concentration (50 ml EPN-suspension on 10 leaflets * 4 times). Water without nematodes was sprayed on 40 leaflets as a control treatment. Thereafter, each leaflet was placed singly in a plastic box (10 cm L * 6 cm W * 6 cm H) with their petioles placed into moistened Peat Moss to keep the leaf fresh (Fig. IV.2). All plastic boxes were incubated at 25 °C for 72 h (Fig. IV.3). Subsequently, the leaflets were examined to determine dead and living larvae per leaflet, and their numbers in the ten leaflets treated with the 50 ml EPN suspension were counted together to form one replicate (on average 30 larvae/replicate). The whole experiment was repeated three times at different time points during the years 2016 and 2017.

Figure IV.2: Plastic box with a tomato leaflet infested with *Tuta absoluta* 4th instar larvae inside mines. The leaflet was treated with a suspension containing *Steinernema carpocapsae* BA2.

Figure IV.3: Plastic boxes containing tomato leaflets infested with *Tuta absoluta* 4th instar larvae treated with *Steinernema carpocapsae* BA2.

IV.3.5. Statistical data analysis

IV.3.5.1. Sand bioassay

The objective of the bioassay was to investigate the efficacy of four nematode isolates against *T. absoluta* larvae. The comparison among isolates within different application doses was assessed. Each application dose had three replicates. Each replicate consisted of the number of dead and living larvae which had been treated at the same time by the nematode suspension. The treated larvae were 40 larvae per replicate. The statistical analysis and graphical presentation of results were performed using R software version "3.4.2" (R Core Team, 2017). The differences among the nematode isolates and doses were analyzed. Analysis of variance (ANOVA) was applied using function "aov". Models were simplified and selected by using the Akaike's Information Criterion (AIC) and drop1 function with "F" test. The fitted model was with EPN isolate and trial as explanatory variables and larval mortality (%) as a response variable. In the case of both doses of 10 and 20 nematodes/larva, one model was fitted with EPN isolate as explanatory variable and larval mortality (%) as a response variable. In the case of doses of one and five nematodes/larva, one model with EPN isolate, trial, and the interaction between EPN isolate and trial as explanatory variables and larval mortality (%) as a response variable. Model residuals were visually checked out, and dispersion was checked as well. The package 'emmeans' (Lenth, 2019) was used to calculate the estimated marginal means, posthoc tests at 0.05 significant level by the Tukey method, and 95% confidence intervals.

IV.3.5.2. Leaf bioassay

The objective of the bioassay was to compare the concentration-mortality response among four isolates and to select the most efficient one on *T. absoluta* larvae on tomato leaves. The statistical analysis and graphical presentation of results were performed using R software version "3.4.2" (R Core Team, 2017). Data of proportion were considered (number of dead larvae versus the number of living larvae in each replicate) and consequently analysis of Deviance, assuming a general linear model (GLM) was applied. One GLM with binomial data distribution and a logit link was fitted with EPN isolate, log (concentration), trial, and the interaction among isolate, log (concentration), and trial as explanatory variables and number of present dead and living larvae as a response variable. Model suitability was visually checked by plotting the standardized residuals versus the fitted values, and dispersion was checked as well. Likelihood-Ratio-Test (LRT) was used following the model simplification to test the significance of the explanatory variables. Lethal concentrations causing 50% and 90% larval mortality (LC_{50} and LC_{90}) and their confidential limits for the four isolates were calculated using the "MASS" package (Venables and Ripley, 2002). The dose-response curves were plotted using 'ggplot2' package (Wickham, 2016).

IV.4. RESULTS

IV.4.1. Sand Bioassay

The four isolates most efficient against lepidopteran larvae were explored in sand bioassays on *T. absoluta*. No larval mortality was recorded in the control. There were significant differences among the tested isolates at the application dose of 20 IJs/larva (Table IV.1) (aov (Mortality ~ Isolate): df=3, F value=18.13, p<0.001). All the tested isolates were highly virulent against *T. absoluta* larvae in this bioassay, as they achieved above 90% larval mortality. The larvae were also highly susceptible when treated with the lower dose of 10 IJs/larva with significant differences occurred among the tested isolates (Table IV.1) (aov (Mortality ~ Isolate): df=3, F value=6.22, p=0.017). At the dose of 5 IJs/larva, the trial time has an influence on the larval mortality according to the fitted model (aov (Mortality ~ Isolate * Trial)) (isolate: df=3, F value=36.87, p=0.002, Trial: df=1, F value=25.6, p=0.007, isolate: trial: df=3, F value=12.53, p=0.016). *S. abbasi* abb caused the highest mortality in *T. absoluta* larvae (Table IV.1). Using one nematode on one larva caused lower larval mortality, but the effect of the isolates was not significantly different (Table IV.1) (aov (Mortality ~ Isolate * trial: F=0.7, df=3, p>0.05). These results suggest that all the selected isolates were efficient against *T. absoluta* larvae by direct exposure in the sand bioassay.

Table IV.1: Mortality (mean±SD, %) of *Tuta absoluta* (4th instar) after exposure to different doses of four EPN isolates in standard sand bioassay. BA2, J7: *Steinernema carpocapsae*, Sf: *S. feltiae*, abb: *S. abbasi*. *Mean values followed by different letters in the same column are statistically different for each application dose according to Tukey's test (p < 0.05).

EPN Isolate	Nematode dose (IJ/larva)			
	1	5	10	20
	Mean±SD* (%)	Mean±SD* (%)	Mean±SD* (%)	Mean±SD* (%)
BA2	34.1±22.2 a	73.3±9.4 b	94.1±2.2 ab	100 a
Sf	25.8±14.4 a	72.5±1.6 b	94.1±3.8 ab	98.3±1.1 a
abb	45.83±3.8 a	93.3±2.2 a	100 a	100 a
J7	52.5±10 a	83.3±5.5 c	88.3±2.2 b	91.6±2.2 a

IV.4.2. Leaf bioassay

All tested isolates successfully infected *T. absoluta* larvae on tomato leaves, whether inside or outside their mines (Fig. IV.4). The nematode efficacy was evaluated by calculating the lethal concentrations of applied IJs. The values of LC_{50} ranged between 44 IJs/ml for EPN isolate *S. carpocapsae* BA2 to 112 IJs/ml for *S. feltiae* Sf (Table IV.1). In Figure IV.5, the relationship between the concentration of IJs/ml (log-transformed) and death probability (larval mortality (%)) including confidence intervals (95%) indicated that the isolate BA2

achieved the highest larval mortalities and was significantly separated from the other three isolates. To reach 90% mortality of 4th instar larvae (LC_{90} values), the application of 305, 1179, 714, and 3624 IJs/ml for nematodes of BA2, Sf, abb, and J7, respectively, was necessary (Table 1). Corresponding confidence intervals ranged between 338 and 392 for BA2, 885 and 1570 IJs/ml for Sf, 511 and 997 for abb, and 2374 and 5532 for J7. Again, BA2 was clearly separated from the other isolates and achieved highest mortality on the 4th instar larvae of *T. absoluta*.

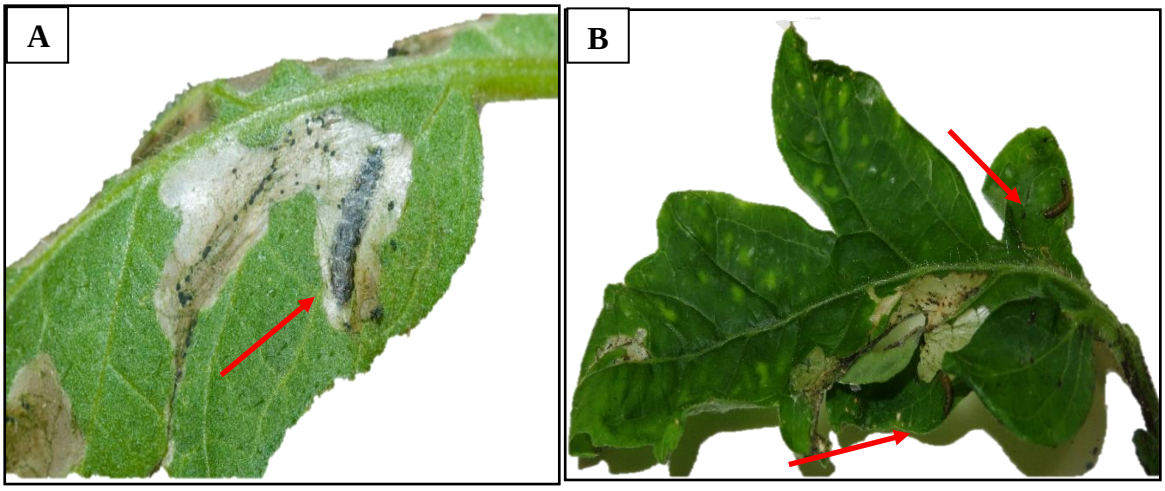

Figure IV.4: *Tuta absoluta* larvae infected by *Steinernema carpocapsae* BA2 infective juveniles applied on tomato leaves. (A) Infected larva inside the mine. (B) Infected larvae outside the mines.

Table IV.2: The lethal concentrations causing 50% and 90% larval mortality (LC_{50} and LC_{90}) and the confidence intervals of four nematode isolates (*Steinernema carpocapsae* BA2 (BA2), *S. feltiae* (Sf), *S. abbasi* (abb), and *S. carpocapsae* J7 (J7)) tested against *Tuta absoluta* 4th instar larvae in leaf bioassay. LCL=lower 95% confidence limit, UCL=upper 95% confidence limit.

Nematode isolate	Lethal concentration	(Mean±SE) (IJ/ml)	LCL (IJ/ml)	UCL (IJ/ml)	Number of treated insects
BA2	LC50	43.58±1.08	36.88	51.50	2158
	LC90	305.18±1.13	237.63	391.92	
Sf	LC50	112.46±1.07	98.34	128.60	2599
	LC90	1178.72±1.15	885.07	1569.79	
Abb	LC50	82.40±1.08	69.60	97.54	1978
	LC90	713.78±1.18	510.90	997.22	
J7	LC50	102.73±1.12	81.61	129.33	2193
	LC90	3623.71±1.24	2373.55	5532.34	

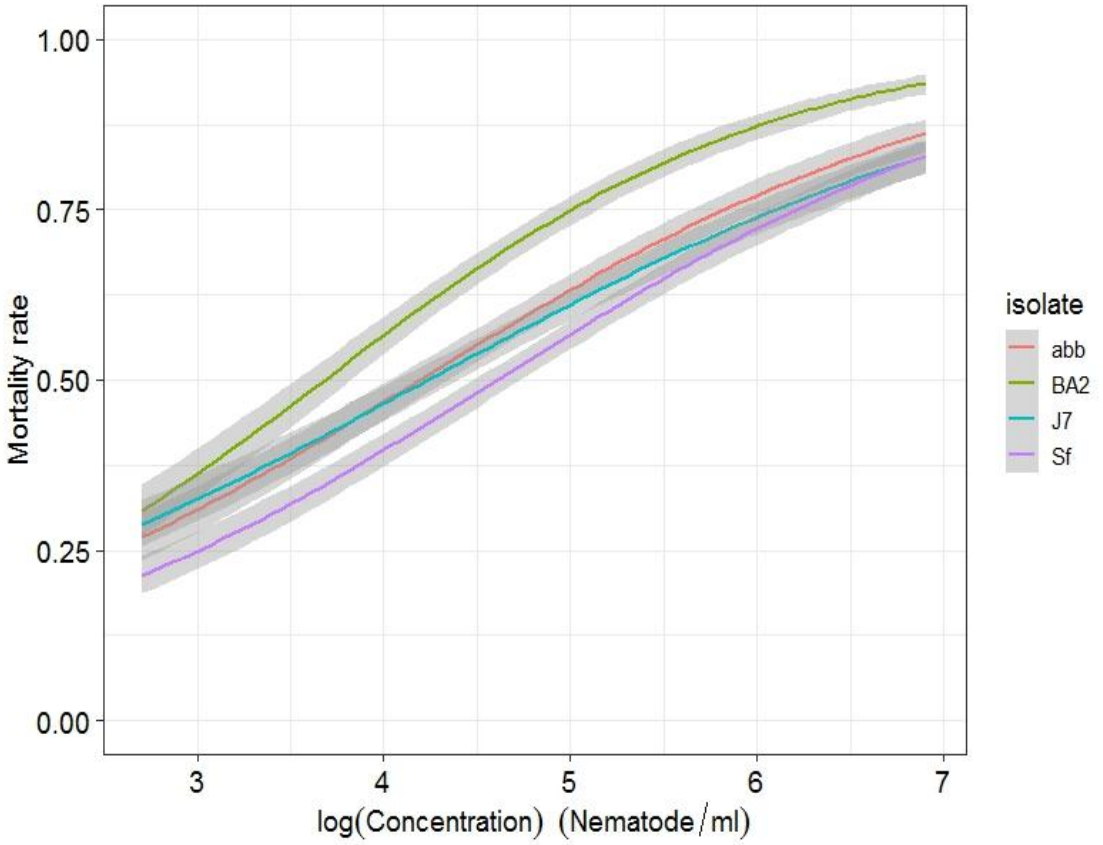

Figure IV.5: The relationship between the nematode concentrations and the *Tuta absoluta* larval mortality rate caused by the tested four nematode isolates in leaf bioassay.

IV.5. DISCUSSION

In the current investigation, the most efficient EPN isolates from a previous screening against lepidopteran larvae (Chapter II) were tested again and more profound against *T. absoluta* larvae in replicated sand and leaf bioassays. The tomato leafminer *T. absoluta* is the prevailing and most destructive pest on tomato in Egypt and was accordingly selected as the target pest to develop a control system against ETPC. *T. absoluta* larvae are smaller in size in comparison to Noctuid larvae, and develop through only four larval instars before pupation. In the previous screening (Chapter II), four EPN isolates were selected as top candidates, as they were highly virulent against all target insects. In the current study, the efficacy of these EPN isolates was confirmed once more on *T. absoluta* larvae under similar conditions. According to the obtained results, all the selected isolates were efficient against *T. absoluta* larvae under controlled conditions by direct exposure in the sand bioassay. Under these conditions, there were no significant differences among the tested isolates. Nevertheless, the efficacy of EPNs was proven against *T. absoluta* larvae under laboratory conditions by other studies (Batalla-Carrera et al., 2010; Garcia-del-Pino et al., 2013; Van Damme et al., 2016). High mortality levels were recorded by *S. carpocapsae* (86.6%) and *S. feltiae* (100%) when applied 50 IJs/cm^2 against *T. absoluta* larvae in Petri dishes filled with sand under laboratory conditions (Batalla-Carrera et al., 2010). In the same direction, high control levels of 52.3% for *S. feltiae*, 100% for *S. carpocapsae*, and 96.7% for *H. bacteriophora* were obtained by applications of 50 IJs/cm^2 on soil surface inside plastic boxes against *T. absoluta* larvae that

drop to the soil for pupation (Garcia-del-Pino et al., 2013). According to the obtained current results and the previously reported results, EPNs are highly virulent against *T. absoluta* larvae on soil under optimized laboratory conditions to ensure high nematode performance.

In leaf bioassays, the efficacy of four nematode isolates was tested against *T. absoluta* larvae on tomato leaves. These bioassays were conducted under controlled laboratory conditions to test the nematode ability to infect the insect larvae on tomato leaves. Testing the nematodes against the insect on leaves revealed to be a necessary step between sand bioassays and experiments on plants. Also, using the whole tomato leaflet instead of leaf disks as done in other studies (Van Damme et al., 2016) made the conditions more challengeable for the nematodes to find the larvae by allowing the insect larvae to escape, as it occurs under natural conditions.

All the tested nematode isolates were able to infect *T. absoluta* larvae inside their galleries. Similar observations were made in leaf bioassays conducted by Batalla et al (2010). They recorded high infection levels (77.1-91.7%) in *T. absoluta* larvae inside the galleries after treated by *S. carpocapsae*, *S. feltiae*, and *H. bacteriophora* in comparable leaf bioassays. Also, *H. bacteriophora* (HP88), and *Steinernema monticolum* (Stock, Choo & Kaya) caused high mortality (80-100%) of *T. absoluta* larvae in laboratory leaf bioassays performed by Shamseldean et al. (2014). In contrast, Türköz and Kaskavalci (2016) found that the application of *S. feltiae* against *T. absoluta* larvae inside the mines resulted in low larval mortality (19%).

The lethal concentrations were calculated to select the most virulent nematode isolate among the tested isolates against *T. absoluta* larvae. The results showed that *S. carpocapsae* BA2 was the most efficient isolate as it obtained the lowest LC_{50} value among the tested isolates. The values of LC_{90} supported the previous results as it showed that *S. carpocapsae* BA2 is the most efficient isolates against *T. absoluta* larvae. The results are in agreement with the results from Van Damme et al (2016) who tested three nematode isolates (*S. carpocapsae*, *S. feltiae*, and *H. bacteriophora*) and found that *S. carpocapsae* was the most efficient isolate against *T. absoluta* 4^{th} instar larvae. Also, *S. carpocapsae* and *S. feltiae* obtained higher larval mortality levels than the nematode of *H. bacteriophora* but with no significant difference (Batalla et al., 2010). There was no overlap between the confidence interval curves of *S. carpocapsae* BA2 and the other isolates. The results pointed to *S. carpocapsae* BA2 isolate as the most efficient isolate among the tested isolates on *T. absoluta* larvae in leaf bioassays. Therefore, the isolate of *S. carpocapsae* BA2 was selected to be used in further experiments to examine application methods (Chapter V).

The results of both bioassays showed that the significant differences among the nematode isolates appeared only when the conditions were more challengeable. The isolate of *S. carpocapsae* BA2 was more virulent than the other three isolates under leaf bioassay conditions. The performed bioassay conditions created a permanent film of water on the leaflets, which might be contributed to the significant differences in the achieved mortality rates. Moreover, the differences in host-seeking behavior and mobility on leaf surfaces among the tested isolates could affect their efficacy under these conditions.

Collectively, these results confirmed that the selected EPN isolates are able to infect *T. absoluta* larvae on tomato leaves. Also, the foliar application using these isolates against ETPC is possible. On the other hand, this creates new challenges: to know more about optimized applications, e.g. by formulation and application schedules is required. In the following chapter, further investigations will be conducted on optimizing application strategy against the target pest on tomato plants.

Chapter V: Optimizing foliar application of entomopathogenic nematodes against the Tomato leafminer, *Tuta absoluta*

V.1. ABSTRACT

The tomato leafminer moth, *Tuta absoluta* is a serious pest on tomatoes. The larvae attack the above-ground parts of the plant. Foliar application of entomopathogenic nematodes (EPNs) was investigated towards an effective biological control method for this pest. The trials were conducted to select suitable EPN concentrations and adjuvants to improve the efficacy and persistence of EPNs in foliar applications on tomatoes. Four isolates (*Steinernema carpocapsae* BA2, *S. feltiae* Sf, *S. abbasi* abb, and *S. carpocapsae* J7) were applied in several concentrations and sprayed once or twice on infested tomato plants within 24 h. Applying the nematode suspensions twice caused higher mortalities of *T. absoluta* 4th instar larvae than sprayed once with a double concentration. EPN isolates were able to cause above 80% larval mortality within the highest concentration (20000 infective juveniles (IJs) twice); only *S. abbasi* abb evoked here low mortality of only 32.5%. As the next step, different formulations were explored based on a suspension of *S. carpocapsae* BA2 in a concentration of 5000 IJs/ml and various adjuvants and applied twice on infested tomato plants. The adjuvants Xanthan, Nemaperfect®, or Chitosan in EPN suspensions significantly increased the mortality in *T. absoluta* larvae. The previous three adjuvants increased mortality from 70% (water control) up to 88% (Xanthan). If it is available and economically affordable, adding adjuvants can be recommended to improve foliar applications of EPN against *T. absoluta* on tomato.

V.2. INTRODUCTION

EPNs are soil-dwelling organisms so that the free-living developmental stages - the IJs - naturally search and infect insect hosts in soil. For that reason, EPNs have mainly been used as bio-pesticides against insect pests inhabiting the soil or spending part of their life in the soil. Nevertheless, applications of EPNs on upper parts of the plant, e.g. stems and leaves, have also been tested against a range of pests in several studies (Baur et al., 1997; Brusselman et al., 2012; Mason et al., 1998; Schroer and Ehlers, 2005).

In particular, foliar application of EPNs showed potential success for control of some insect pests such as *Spodoptera littoralis* and *Agrotis ipsilon* (Abonaem, 2013), *S. littoralis* and *Helicoverpa armigera* (Navon et al., 2002), *S. exigua* and *Plutella xylostella* (Mahmoud, 2014) and also against leafminers such as *Liriomyza trifolii* (LeBeck et al., 1993; Tomalak et al., 2005) and *Liriomyza huidobrensis* (Williams and Walters, 2000) (Diptera: Agromyzidae), and *T. absoluta* (Batalla-Carrera et al., 2010).

The success of EPNs foliar application depends mainly on the nematode's infectivity and their persistence on plant foliage. Infectivity depends on the nematode species and their ability to invade and kill the target insect pest. Persistence (survival, mobility) of EPNs on foliage experiences limiting factors such as desiccation, UV radiation, and high temperature (Koppenhöfer, 2000; Georgis et al., 2006; Lewis et al., 2015). Therefore, different formulations were investigated in some research efforts by adding adjuvants to the nematodes

suspension before the foliar application (Head et al., 2004; Schroer and Ehlers, 2005). The adjuvants increase EPNs persistence by serving as humectants, surfactants, and adhesions. The humectant adjuvants decrease evaporation and keep a film of water on the leaf surface, making EPN mobility for host searching possible. The surfactants reduce the surface tension of the applied suspension droplets and improve wetting. Adhesions stick the nematode suspension on the leaf surface and reduce droplets runoff.

Several studies have examined different formulations and application techniques against foliar insect pests. Adding the antidesiccants Glycerol or Folicote® with *S. feltiae* against *H. armigera* resulted in 75% and 95% larval mortality (Glazer and Navon, 1990). In another study, a formulation consisting of *S. feltiae* and calcium-alginate gel caused 89% mortality in *S. littoralis* and *H. armigera* larvae on tomato under greenhouse conditions (Navon et al., 2002). Further trials were conducted using surfactant-polymer-formulation (0.3% Rimulgan® together with 0.3% xanthan) to increase EPNs persistence on cabbage leaves to control the diamondback moth larvae *Plutella xylostella* (Schroer and Ehlers, 2005). Moreover, adding Barricade® (a sprayable fire-gel, which is originally used to protect houses or trees from fires) to *S. carpocapsae* suspension improved their efficacy against the lesser peachtree borer, *Synanthedon pictipes* (Grote & Robinson) (Shapiro-Ilan et al., 2010). Furthermore, adding the sprayable fire-gel with EPN applications enhanced their efficacy against the codling moth, *Cydia pomonella* (L.) in apple tree trunks (Lacey et al., 2010). In another trial, a formulation of *S. carpocapsae* with chitosan was applied against the red palm weevil, *Rhynchophorus ferrugineus* (Olivier) (Llacer et al., 2009). Trials using EPNs with or without adjuvants were applied on *T. absoluta* as well. Adding 0.05% Addit® to nematode suspensions achieved 87-95% mortality of this pest on potted tomato plants under greenhouse conditions (Battalla et al., 2010). The adjuvants SilwetL-77® and Addit® improved the control ability of EPNs against *T. absoluta* larvae even on hanged tomato disks (Van Damme et al., 2016). Field trials were conducted using EPNs against *T. absoluta* in different countries (Gözel and Kasap, 2015; Shamseldean et al., 2014) with variable results according to EPN species and season. But in general, these studies demonstrated partial success against foliar insect pests, if appropriate conditions were met.

The following experiments focused on the selection of suitable concentrations of effective EPN isolates and improvement of their infectivity by adding adjuvants. Sufficient prolongation of their persistence on tomato foliage is needed to control *T. absoluta* and other tomato pests in Egyptian crop growing conditions. First, it was necessary to find a suitable dosage that achieved at least 50% mortality of *T. absoluta* larvae on tomato plants, and then to elaborate an even higher effect by optimizing application frequency. Subsequently, a further increase in efficacy was to be achieved by testing and selecting an appropriate adjuvant.

V.3. MATERIALS AND METHODS

V.3.1. Preparation and infestation of tomato plants

Tomato plants (Tomato Alissa F1, Nunhems Netherlands BV, Nunhem, Netherlands) were grown as described in the previous chapters. Two different methods were used to create a standard infestation. The first method allowed the *T. absoluta* moths to deposit eggs

simultaneously on 35 tomato plants (six leaves old). The plants were placed in an Insect Rearing Tents (BugDorm-2120F) (60 cm L * 60 cm W * 60 cm H) with about 200 freshly emerged *T. absoluta* moths for two days at 24-26 °C, 16 h light: 8 h dark cycle. Then, plants with eggs were transferred into a new Tent and held at the same conditions until eggs had hatched and developed into 4th instar, usually after two weeks, to be ready to use in the trials. On average, there were about 30 larvae per plant. The second infestation method was performed by placing 30 larvae (3rd instar) on the top side of the leaves on each tomato plant (six leaves old). The larvae started to mine and fed on the tissues of the leaves. Experimental conditions were as described above, but here larvae had developed into the 4th instar after two days and the plants were used in the bioassays.

V.3.2. Experiment 1: EPN concentration and application frequency on tomato foliage

The EPN isolates *S. carpocapsae* BA2 and J7, *S. feltiae* Sf, and *S. abbasi* abb had been shown to be most effective against larvae of the leafminer moth in previous bioassays (Chapter IV). The nematodes were applied at concentrations of 2500, 5000, 10,000, and 20,000 (IJs)/ml in water. A control treatment was performed by applying only water. Plants, infested by the 4th larval instar of *T. absoluta,* were treated once or twice within 24 h. Treatment was done by spraying a volume of 200 ml of each EPN concentration or water on four infested plants until runoff, using a hand sprayer (volume 500 ml). The four plants were treated one after another until the sprayer was empty. The nematode suspension was applied on the whole plant and leaves were treated from both sides as well. Each EPN concentration was applied once on four plants or, as an alternative treatment, the same amount of nematodes was applied on other four plants in two sprayings within 24 h. The plants were placed on a table in a controlled room at 25 °C and long-day conditions for 72 h. Thereafter, leaves were examined to count dead and live *T. absoluta* larvae per plant. This bioassay was performed four times in case of BA2 (the most efficient EPN in previous trials) and twice in case of other isolates, each at different time points.

V.3.3. Experiment 2: Optimizing the EPN formulation by adjuvants

Seven adjuvants were investigated to improve the effect of EPN applications on *T. absoluta* larvae (4th instar) on tomato plants. The nematode isolate used in this experiment was the most efficient isolate according to the results of the previous experiments. The tested adjuvants are listed in Table V.1. Nematode suspension in water only was used as a control treatment. The formulation of each adjuvant and the nematode suspension were prepared just before the application. For that, formulations were prepared in a volume of 250 ml. First, the double concentration of each adjuvant was prepared in 125 ml, and then 125 ml EPN suspension at a concentration of 10,000 IJs/ml was added. The final suspension was a volume of 250 ml containing 5000 IJs/ml and the suggested adjuvant concentration.

Tomato plants were prepared as described above. The plants (eight leaves old) were infested by adding *T. absoluta* 3rd instar larva (on average 30 larvae per plant) two days before the experiment. The larvae became 4th instars by the day of the experiment. Nematodes

of isolate *S. carpocapsae* BA2 were applied in a concentration of 5000 IJs/ml in a volume of 200 to 250 ml on four or five tomato plants, respectively. Each group of plants was treated again after 24 hours by the same formulation. The plants were placed in a controlled room at 25 °C, long-day conditions, for 72 h. Air humidity was not controlled. Then, plants and their pots were carefully examined and numbers of dead and living larvae were counted. The entire experiment was performed three times at different time points during 2017 and 2018. At first, each formulation was applied on five plants in 2017, whereas four plants were used for each formulation during the second and the third time in 2018.

Table V.1: List of the tested adjuvants.

Commercial name	**Material**	**Function and common use**	**Producer**	**Concentration (%)**
Squall®	Polymer	Adhesive and anti-drift	GreenA B.V.	0.5
Addit®	undisclosed surfactant + vegetable oil	Surfactant	Koppert	0.25
Nemaperfect®	Surfactant+Polymer	Emulsifier, Thickener	E-nema	0.3
Xanthan gum	Fermentation-derived biopolymer from the bacterium *Xanthomonas campestris*	Humectant, Thickener	Spinnrad GmbH	0.3
Chitosan	A polymer of glucosamine sugars	Thickener, Adhesive	ChiPro GmbH	0.3
CMC	Carpoxymethelcellelouse	Thickener, Adhesive		0.3
Sorbitol	Sorbitol	Humectant		0.3

V.3.4. Data analysis

V.3.4.1. Experiment 1: EPN concentration and application frequency on tomato foliage

The bioassay aimed to select an appropriate application method of four nematode isolates against *T. absoluta* larvae on tomato plants. The nematode efficacy at different concentrations and different application times was analyzed. Each concentration was applied on four tomato plants infested with about 30 larvae. The numbers of dead and living larvae per plant were counted, and larval mortality (%) was calculated. Each plant was considered as one replicate. The nematodes were applied once or twice within 24 h. In case of *S. carpocapsae* BA2, the experiment was conducted four times at different time points. However, in case of the other three isolates, the experiment was conducted only twice at different time points. The statistical analysis and graphical presentation of results were performed using R software version "3.4.2" (R Core Team, 2017). Analysis of variance (ANOVA) was applied using function "aov". Models were simplified and selected by using the Akaike's Information Criterion (AIC) and drop1 function with "F" test. Model suitability was visually checked by plotting the standardized residuals versus the fitted values, and dispersion was checked as well. Shapiro-Wilk's normality test (Shapiro and Wilk, 1965) was used to assess the normal distribution, and Levene's test (Fox and Weisberg, 2011) was used to test homogeneity of variances. The residuals were normally distributed ($p>0.05$), and the homogeneity of variances was present ($p>0.05$). The package "emmeans" (Lenth, 2019) was used to calculate the estimated marginal means, posthoc tests at 0.05 significant level by the Tukey method, and 95% confidence intervals. The results were plotted using 'ggplot2' package (Wickham, 2016).

V.3.4.2. Experiment 2: Optimizing the EPN formulation by adjuvants

The aim of the bioassay was to test different adjuvants added to nematode suspension against *T. absoluta* larvae on tomato plants. The nematode efficacy with different adjuvants was analyzed. Seven different adjuvants were tested and compared with nematodes in water only. Each nematode suspension was applied on four tomato plants infested by about 30 larvae. The nematodes of *S. carpocapsae* BA2 were applied at a concentration of 5000 IJs/ml twice within 24 hours. The experiment was conducted three times at different time points. The numbers of dead and living larvae per plant were counted and larval mortality (%) was calculated. Each plant was considered as one replicate. The statistical analysis and graphical presentation of results followed the same procedures as described for experiment 1 (V.3.4.2).

V.4. RESULTS

V.4.1. Experiment 1: EPN concentration and application frequency on tomato foliage

Bioassays aimed to determine the effects of concentration and frequency of application on the efficacy of each EPN isolate separately, therefore the results were analyzed and presented one by one. The application of water without nematodes as a control did not result in larval mortality in *T. absoluta*. Therefore, the control was not included in the statistical analysis.

V.4.1.1. *Steinernema carpocapsae* (BA2)

Nematode IJs successfully infected *T. absoluta* larvae on the tomato plants, even inside their galleries (Fig. V.1). The nematode application at different concentrations resulted in 29-81% larval mortality. Significant differences were found when applying EPNs once and twice with the same concentration (Fig. V.2) and, more important, that the two-fold application reached higher mortality than one application of the two-fold concentration. Applying the concentration of 2500 IJs/ml twice resulted in 58.71% larval mortality, whereas applying the concentration of 5000 IJs/ml once caused 39.92% larval mortality. The application of 5000 IJs/ml twice caused 65.4% larval mortality, at the same time applying 10,000 IJs/ml once caused 53.97% larval mortality. Using 10,000 IJs/ml twice caused 74.74% larval mortality, and using 20,000 IJs/ml once caused 69.6% larval mortality.

Figure V.1: Infected *Tuta absoluta* larvae treated with *Steinernema carpocapsae* IJs on tomato plants.

The results for the mortality of *T. absoluta* larvae analyzed using ANOVA, showed significant differences among the applied concentrations (Fig. V.2). The fitted model was (Model = aov (Mortality ~ Concentration + Application + Trial + Concentration: Application)) (Table V.2). There were significant differences among the four concentrations when applied once. In contrast, there were interactions between the concentrations when applied twice. No significant difference was noted between 2500 and 5000 IJs/ml when applied twice. Also, there was no significant difference in the mortality between the concentrations of 5000 and 10,000 IJs/ml when applied twice. The larval mortality caused by applying the nematodes in a concentration of 10,000 or 20,000 IJs/ml twice was not significantly different.

The mortality of *T. absoluta* larvae showed no significant difference among the concentrations of 5000 IJs/ml twice, 10,000 IJs/ml twice, and 20,000 IJs/ml once (Fig. V.2). Only the highest application rate (20,000 IJs/ml twice) resulted in significantly higher larval mortality level than 5000 IJs/ml twice. As a consequence, the application of 5000 IJs/ml twice was used in the next experiment to select an adjuvant.

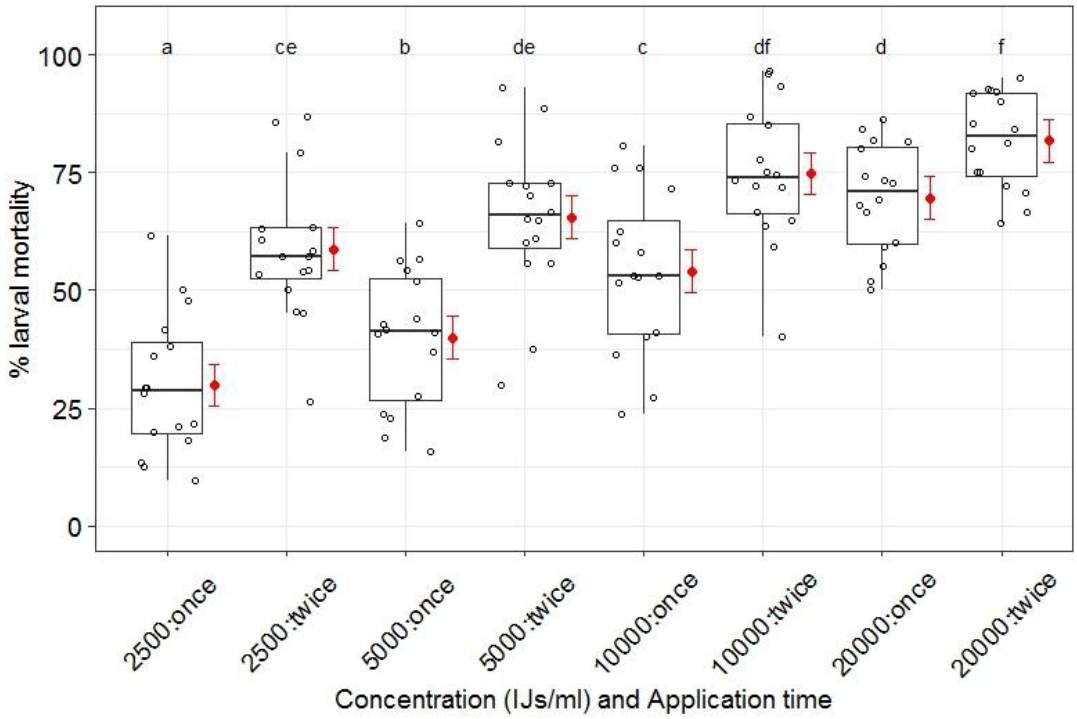

Figure V.2: Larval mortality (%) of *Tuta absoluta* in relation to treatment of *Steinernema carpocapsae* BA2 applied in different concentrations once or twice. Boxplots are shown with individual observed values (n=16) as jittered points. The median is displayed by the horizontal line inside the boxplot. The estimated marginal means are displayed by the red dots and 95% confidence intervals estimated from the fitted mixed model. The letters at the top indicate significant differences among the tested treatments according to posthoc tests ($p < 0.05$).

Table V.2: The significant explanatory variables affecting *Tuta absoluta* larval mortality (%) obtained by *Steinernema carpocapsae* BA2 according to the fitted ANOVA model in R.

Variables	Df	F value	Pr(>F)
Concentration	3	71.934	<0.0001
Application	1	182.381	<0.0001
Trial	3	62.576	<0.0001
Concentration:Application	3	5.047	0.00253

V.4.1.2. *Steinernema feltiae* Sf

The application of *S. feltiae* resulted in larval mortality of *T. absoluta* ranged between 38.04±10.08% and 83.67±4.6% (Fig. V.3). The results indicate that dividing the number of nematodes and applying it twice within 24 hours is better than applying the same nematodes amount once (Fig. V.3). The fitted model was (Model = aov (Mortality ~ Concentration + Application + Trial + Concentration: Trial + Application: Trial)) (Table V.3). The achieved larval mortalities by the application of 5000 IJs/ml twice (67.46%) and 20,000 IJs/ml once (66.91%) were statistically equal but significantly higher than that achieved by 10,000 IJs/ml once (52.61%). The larval mortality increased significantly when the nematode concentration was 10,000 IJs/ml twice (79.42%) and 20,000 IJs/ml twice (83.67%). The results of *S. feltiae* Sf clearly support the results of *S. carpocapsae* BA2 on tomato plants.

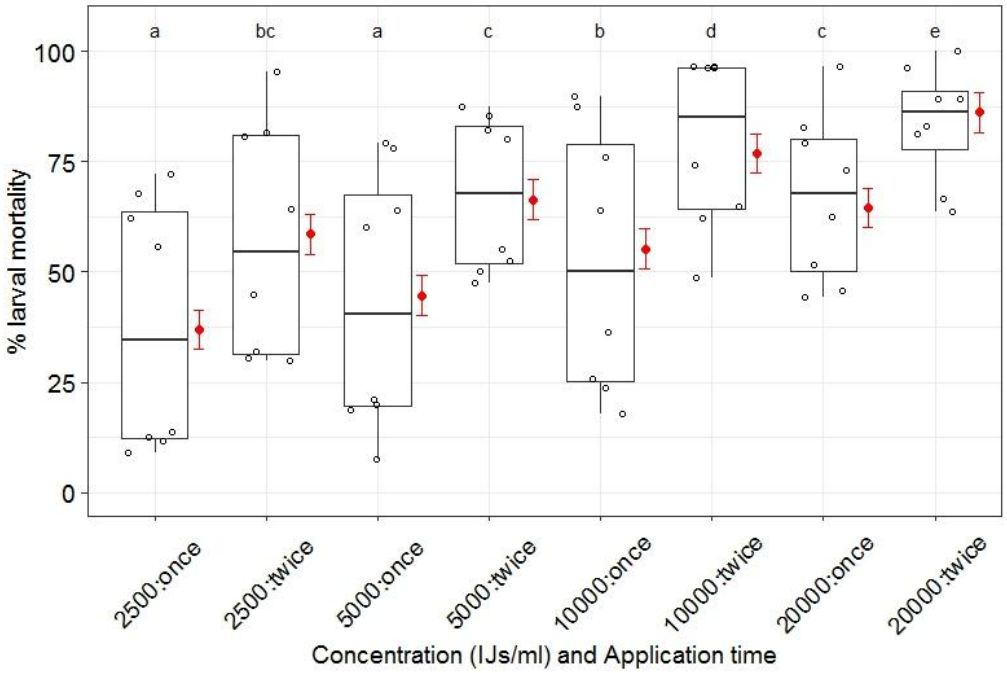

Figure V.3: Larval mortality of *Tuta absoluta* in relation to treatment of *Steinernema feltiae* Sf applied in different concentrations once or twice. Boxplots are shown with individual observed values (n=8) as jittered points. Other explanations see Fig. V.2.

Table V.3: The significant explanatory variables affecting *Tuta absoluta* larval mortality (%) obtained by *Steinernema feltiae* Sf according to the fitted ANOVA model in R.

variables	Df	F value	Pr(>F)
Concentration	3	36.233	< 0.0001
Application	1	117.175	< 0.0001
Trial	1	408.6	< 0.0001
concentration:Application	3	6.363	0.0008
Application:Trial	1	13.353	0.0005

V.4.1.3. *Steinernema abbasi* abb

The results showed that *S. abbasi* abb has poor efficacy against *T. absoluta* larvae on tomato plants (Fig. V.4). The mean mortality in *T. absoluta* larvae ranged between 6.83±5.31 and 32.51±11.02%. The highest mortality was achieved by application of 20,000 IJs/ml twice but was not significantly different from 5000 and 20,000 IJs/ml once. The fitted model was (Model = aov (Mortality ~ Concentration * Application * Trial)) (Table V.4). Nearly all applied concentrations resulted in low mortality compared to other results, and the statistical differences among them were limited. In general, this nematode isolate did not perform well against *T. absoluta* larvae on tomato plants, even with high concentrations.

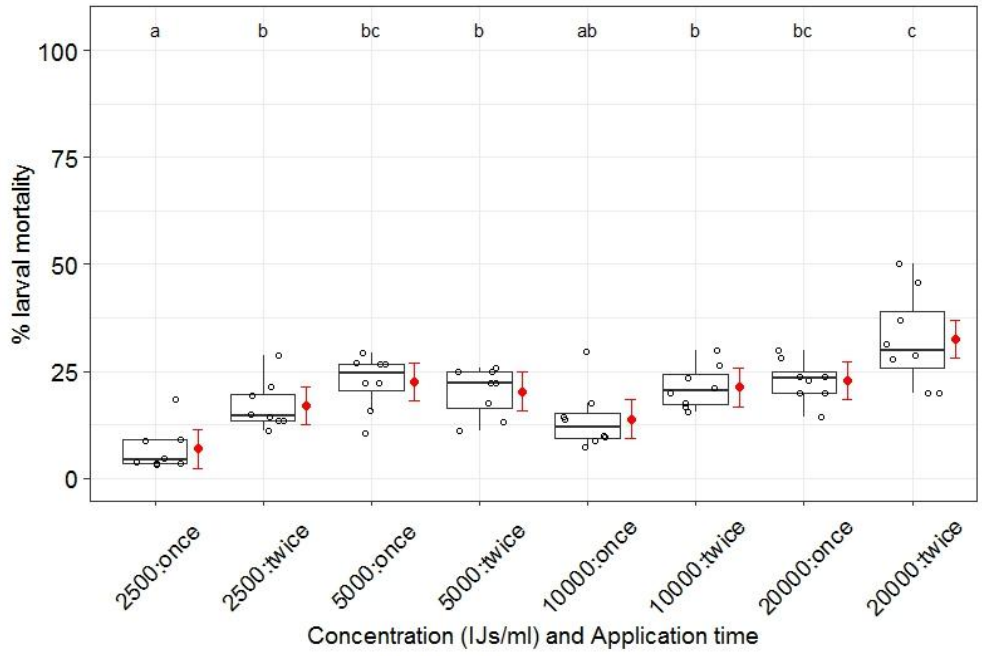

Figure V.4: Larval mortality of *Tuta absoluta* in relation to treatment of *Steinernema abbasi* abb applied in different concentrations once or twice. Boxplots are shown with individual observed values (n=8) as jittered points. Other explanations see Fig. V.2.

Table V.4: The significant explanatory variables affecting *Tuta absoluta* larval mortality (%) obtained by *Steinernema abbasi* abb according to the fitted ANOVA model in R.

variables	Df	F value	Pr(>F)
Concentration	3	17.492	< 0.0001
Application	1	15.522	0.0002
Trial	1	0.715	0.40
concentration: Application	3	3.378	0.02
concentration: Trial	3	1.549	0.21
Application: Trial	1	0.633	0.43
concentration: Application: Trial	3	2.833	0.04

V.4.1.4. *Steinernema carpocapsae* J7

The mean of *T. absoluta* larval mortality ranged between 30.14±4.43% and 86.59±2.1%. The fitted model was (Model = aov (mortality ~ concentration + Application + Trial + Application: Trial)) (Table V.5). The statistical analysis shows that dividing the amount of nematodes and applying it twice within 24 h did not significantly differ from applying the same nematodes amount once (Fig. V.5). Applying the nematodes in a concentration of 5000 IJs/ml twice did not significantly differ from applying it in a concentration of 10,000 IJs/ml once. The application of 10,000 IJs/ml twice did not give significant better results than the application of 20,000 IJs/ml once. The results show that increases the nematode amount increased the nematode efficacy significantly. The nematode application of 2500 IJs/ml twice or 5000 IJs/ml once differs significantly from 2500 IJs/ml once.

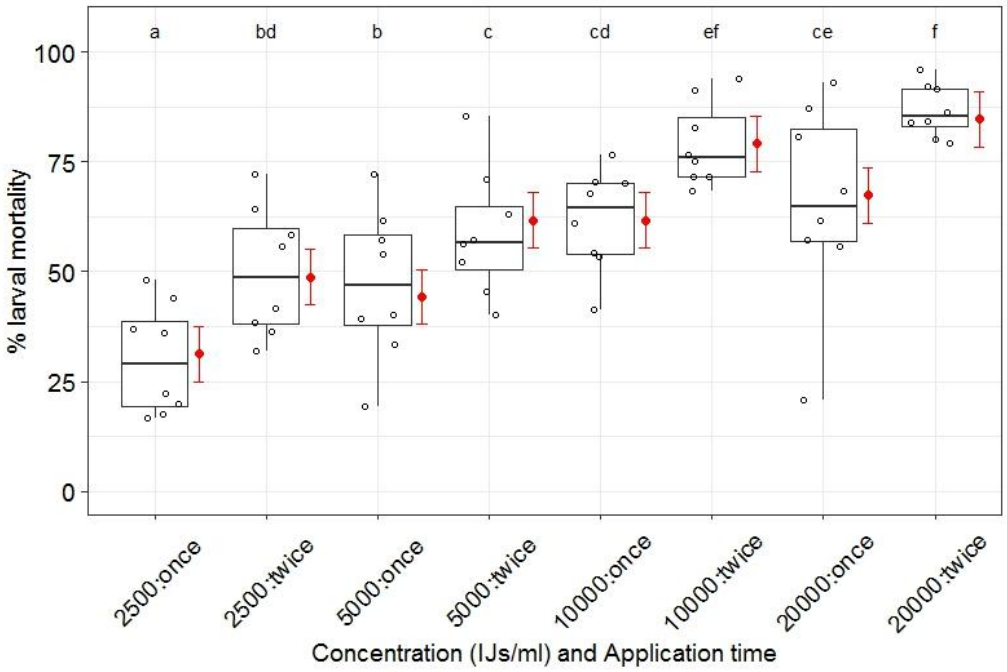

Figure V.5: Larval mortality of *Tuta absoluta* in relation to treatment of *Steinernema carpocapsae* J7 applied in different concentrations once or twice. Boxplots are shown with individual observed values (n=8) as jittered points. Other explanations see Fig. V.2.

Table V.5: The significant explanatory variables affecting *Tuta absoluta* larval mortality (%) obtained by *Steinernema carpocapsae* J7 according to the fitted ANOVA model in R.

variables	Df	F value	Pr(>F)
Concentration	3	34.354	< 0.0001
Application	1	38.111	< 0.0001
Trial	1	30.720	< 0.0001
Application:Trial	1	5.198	0.0264

V.4.2. Experiment 2: Optimizing the EPN formulation by adjuvants

The added adjuvants reduced the surface tension of sprayed water (Fig. V.6). Efficacy of EPN against *T. absoluta* larvae increased by adding any of the adjuvants to the nematodes suspension (5000 IJs/ml of isolate *S. carpocapsae* BA2) compared with the nematodes in water only (Fig. V.7). The highest larval mortality was 87.88% caused by nematodes applied with Xanthan. The lowest mortality (69.54%) was recorded by the application of EPNs in water only.

The fitted statistical model was (Model= aov (Mortality ~ Adjuvant * Trial)) (Table V.6). The analysis of the results obtained using ANOVA and post hoc tests showed that there were no significant differences among Addit®, CMC, Sorbitol, squall, and water (Fig V.7). The adjuvants of Xanthan, Nemaperfect®, and Chitosan increased the nematode efficacy

significantly in comparison with the nematodes in water. There were no significant differences among the nematode efficacy levels with the previous additives. The recorded mortality in *T. absoluta* larvae were 87.88, 85.41, and 81.42%, respectively. Based on visual observation, Nemaperfect® was the best adjuvant to keep the nematodes away from sedimentation in the suspension for the longest time. Based on the previous note, the application of nematodes in Nemaperfect® was chosen to be used in the greenhouse experiment.

Figure V.6: Tomato leaves treated with *Steinernema carpocapsae* in water (left) and with 0.3% Nemaperfect® as adjuvant (right).

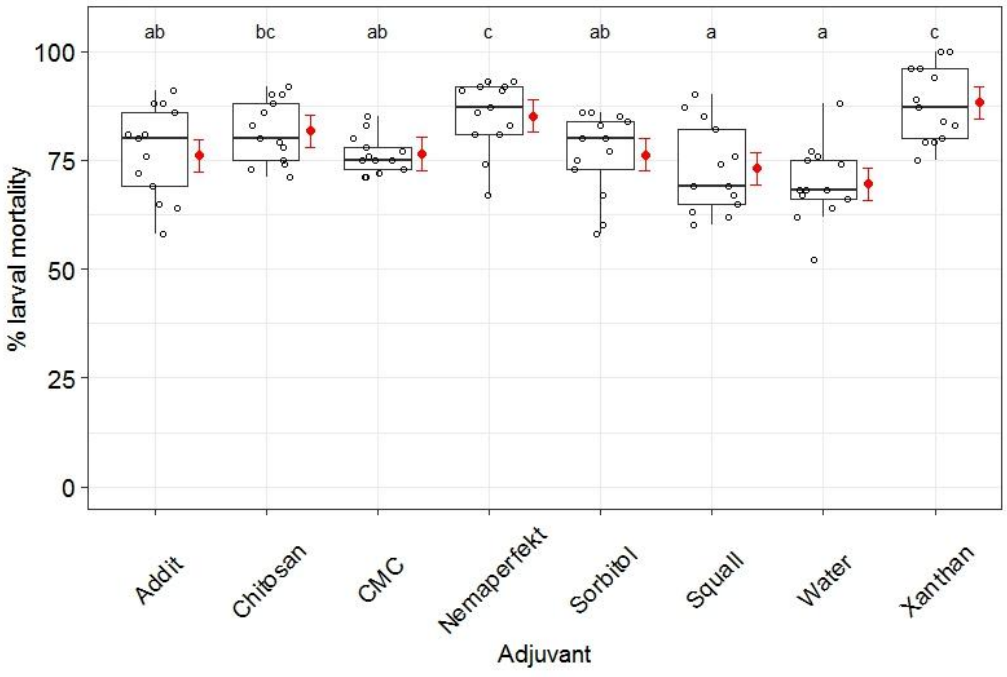

Figure V.7: Larval mortality (%) in *Tuta absoluta* 4th instars treated by *Steinernema carpocapsae* BA2 5000 IJs/ml twice within 24 h with different adjuvants on tomato plants. Boxplots are shown with individual observed values (n=13) as jittered points. Other explanations see Fig. V.2.

Table V.6: The significant explanatory variables affecting *Tuta absoluta* larval mortality (%) obtained by *Steinernema carpocapsae* BA2 with different adjuvants on tomato plants, according to the fitted ANOVA model in R.

variables	Df	F value	Pr(>F)
Adjuvant	7	10.889	0.0001
Trial	2	22.242	0.0001
Adjuvant:Trial	14	2.286	0.0109

V.5. DISCUSSION

Sufficient prolongation of EPN persistence on tomato foliage is desired to control *T. absoluta* and other tomato pests under Egyptian crop growing conditions. EPNs should be applied in sufficient, but affordable amounts on the plants and adjuvants could help to keep costs low while ensuring sufficient effectiveness. The four tested EPN isolates had already shown their ability to infect *T. absoluta* larvae inside their galleries in previous tomato leaf bioassays. Among them, isolate *S. carpocapsae* BA2 was the most efficient one, but all of them were highly virulent against *T. absoluta* larvae following direct exposure to mines of infested tomato leaflets. In the current study, their efficacy was explored under more challengeable conditions on plants with the aim to identify required concentrations and application levels. Dividing the nematode amount and applying it twice within 24 h resulted in mortality levels higher than applying the same amount once. Repeating the application within 24 h boosted the mortality level significantly in most of the tested isolates. The time of application is important to increase the chance of making contact between the nematode and the target insect. EPNs survive only for a few hours on the plant leaves (Wright et al., 2005). For the previous reason, the adjuvants are essential to increase the nematode persistence on leaves. Not only the adjuvants but also repeating the application increased the efficacy of EPNs. The second step was screening different formulations to increase the nematode efficacy on foliar application. As *S. carpocapsae* BA2 was always showing high virulence, so that it was selected to be used in the suggested screening, but at a lower dosage (5000 IJ/ml) to allow differentiation in effectiveness after adding adjuvants. The tested adjuvants were selected based on the lack of harmful effects on mammals, plants or EPNs. Most of them are additives in food or health care products. Based on a preliminary test, no effect was observed on EPN viability (data not shown). Furthermore, they did not cause noticed toxic effects on the treated plants in comparison with the control. The results showed that all the tested adjuvants were able to increase the nematode efficacy in comparison with water only. The nematode efficacies with the adjuvants of Xanthan, Nemaperfect®, and Chitosan were significantly higher than the other adjuvants. The applications of nematodes in Xanthan and Nemaperfect® recorded 87% and 85% larval mortality, respectively. Similar results were observed when applications of nematodes in 0.3% xanthan together with 0.3% Rimulgan® recorded the highest control levels above 90% larval mortality in the diamondback moth larvae (DBM) (Schroer et al., 2005). In leaf disc bioassays, the use of 0.3% xanthan caused a significant increase in the nematode efficacy against DBM (Schroer, 2005). The efficacy of *S. carpocapsae* in a chitosan formulation was about 80% in a curative bioassay and around 98% in a

preventative bioassay against the red palm weevil, *R. ferrugineus* on palms (Llacer et al., 2009). A combination formulation of 0.1% Carboxy-methyl-cellulose (CMC), 0.1% Tween 80, and 0.1% Corn oil was used with *S. carpocapsae* BA2 and *H. bacteriophora* BA1 in foliar application against *S. littoralis* and *A. ipsilon* larvae on corn plants in an open field (Saleh et al., 2015).

Based on visual observations, the adjuvant of Nemaperfect® delayed the nematodes sedimentation before the application. Same results were observed with using 0.3% guar gum, 0.1% alginate, or 0.05% xanthan as they kept the nematodes without sedimentation for 1 h. In another sedimentation test, the nematode sedimentation was delayed when 0.3% xanthan was added to the nematode suspension (Beck et al., 2013). Without stirring, EPNs sediment rapidly on the bottom of the sprayer tank (Brusselman et al., 2010). Adding humectant or dispersant adjuvants to the spray suspension can avoid the rapid sedimentation (Schroer et al., 2005). Adding humectant or adhesion adjuvants to the suspension may reduce spray droplets runoff from the leaves (Schroer et al., 2005).

For further tests, two criteria are decisive: 1. The adjuvant needs to increase the EPNs efficacy significantly. 2. Sedimentation of EPNs in containers before application should be avoided or at least delayed. Taking these criteria into consideration, Nemaperfect® is the most promising adjuvant to be introduced into the EPN based system to control ETPC. Therefore, it was selected for further experiments under greenhouse conditions. The importance of the greenhouse experiment is to give real results. The results of laboratory experiments or under controlled conditions are not always comparable with the results in open fields or greenhouses.

S. feltiae was the most efficient species among four nematode species applied against *T. absoluta* larvae on tomato plants under closed cages and in field conditions (Gözel and Kasap, 2015). These authors reported also high pest mortality (90.7% and 94.3%) through two seasons, even though no adjuvants were added. The application rate was reported to be 50 IJs/cm^2, but no concentration was given. In contrast, *S. carpocapsae* caused only 43% target mortality under the same conditions (Gözel and Kasap, 2015). Field efficacy of *H. bacteriophora* ranged between 60% and 80%, whereas the efficacy of *S. monticolum* ranged between 58% and 67% (Shamseldean et al., 2014).

Obviously high concentrations of EPNs, combined with adjuvants, are really needed to infect the tomato leafminer in the galleries. Field trials using *S. feltiae* (1000 IJs/ml) without any added adjuvants against *T. absoluta* larvae on cultivated tomato crop resulted only in 40-50% larval mortality (Jacobson and Martin, 2011). The application of *S. carpocapsae* (250 IJs/ml) against *T. absoluta* on tomato plants under greenhouse conditions resulted in 12.9% mine reduction (Sabry et al., 2016). When applying *S. feltiae* against *T. absoluta* larvae inside the mines or pupae, the recorded mortality in the larvae inside mines was 19% and in the pupae 7% (Türköz and Kaskavalci, 2016). These results support our results of using high nematode concentrations to obtain an acceptable control level.

The high control levels could be a result of the combination of the nematode species, high concentration, the added adjuvant, and the repeating of the application after 24 h. Also, the application time is important to avoid daylight and high temperature by applying before the sunset or in the early morning.

Chapter VI: Efficacy of EPN isolates against the Tomato leafminer moth, *Tuta absoluta*, under simulated greenhouse conditions

VI.1. ABSTRACT

The tomato leaf miner, *Tuta absoluta* is a destructive pest on tomatoes. It has a great negative impact on tomato production in Egypt. *T. absoluta* attacks the tomato crop in open fields and greenhouses in Egypt. Biological control against *T. absoluta* should be introduced to tomato production to increase food and environment safety. Entomopathogenic nematodes (EPNs) are promising biological control agents. The efficacy of four nematode isolates was tested against *T. absoluta* larvae on tomato plants under greenhouse conditions. The nematodes were applied in 0.3% Nemaperfect® as an adjuvant. The highest larval mortality was 85.5% achieved by the isolate *Steinernema carpocapsae* BA2. However, there were no significant differences among larval mortalities recorded after the application of *S. carpocapsae* BA2, *S. feltiae* Sf, and *S. carpocapsae* J7. Only the fourth nematode isolate, *S. abbasi* abb, was significantly less effective, as its application resulted in 18% larval mortality.

VI.2. INTRODUCTION

Tomato crop is the largest vegetable crop cultivated in Egypt. Egypt is ranked fifth in tomato production worldwide with 6.7 million tons of tomatoes produced annually (FAOSTAT, 2018). The crop occupies about 22% of the cultivated crop area. In Egypt, tomatoes are cultivated in open fields and greenhouses. The crop is planted in winter under greenhouses for exportation, and growers are mainly targeting the European market. For exporting tomatoes to Europe, the growers must fulfill the requirements regarding food safety. The European Union has determined maximum residue levels (MRLs) for chemical pesticides in food products. For this reason, the use of pesticides on tomatoes in greenhouses is limited. The main management techniques are based on natural enemies. Tomato production under greenhouse conditions was under efficient biocontrol conditions until *T. absoluta* appeared in 2009 (Khidr et al., 2013; Goda et al., 2015).

The tomato leaf miner *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae) is a serious pest on tomato crop. *T. absoluta* is originally native to South America. Also, it is present as an economic pest in many countries, especially the main producer's countries such as China, India, Turkey, Spain, and Egypt (Desneux et al., 2011; Guedes and Picanço, 2012; Sankarganesh et al., 2017; Biondi et al., 2018; Mansour et al., 2018). The moths of this pest were firstly detected in Egypt in 2009 (Khidr et al., 2013; Goda et al., 2015). Since then, it has become the most destructive pest on the tomato crop in Egypt. *T. absoluta* larvae result in a significant reduction in tomato yield. The tomato yield decreases yearly since 2009 (FAOSTAT, 2018).

This pest has some features that make it an economic pest in the invaded areas. *T. absoluta* has high reproduction ability as one female moth can lay more than 250 eggs during its life span (Uchoa-Fernandes et al., 1995; Duarte et al., 2015). Moreover, the insect can complete its lifecycle within 25 days and has about 12 generations per year. The previous facts make *T. absoluta* capable to adapt to new occupied areas. Also, it can develop resistance to chemical insecticides. The insect has four larval instars, and the most economic are 3rd and

4th instars. The larvae feed on the above-ground parts of tomato plants. The most favorable parts are leaves, then stems, and fruits. The losses in yield could reach 100% on tomatoes (Desneux et al., 2010, 2011; Biondi et al., 2018).

The main management tactics used against this pest are based on chemical insecticides due to the unavailability of other effective options (Biondi et al., 2018). Using chemical insecticides on tomatoes has some negative effects on food safety and the environment as well. The extensive use of insecticides can make the insect populations develop resistance to insecticides (Campos et al., 2015; Roditakis et al., 2018). Therefore, integrated pest management (IPM) systems based on natural enemies of *T. absoluta* are essential to minimize the negative outcomes of using chemicals. Recently, there were some attempts to use management tactics based on biological control agents. Also, sex pheromone traps play roles in monitoring *T. absoluta* infestation in tomato fields. Some reports showed promising results using parasitoids, predators, and pathogens. Based on laboratory screening, three *Trichogramma* strains were promising for biological control against *T. absoluta* in Europe (Schäfer and Herz, 2020). Application a combination consists of *Bacillus thuringiensis* Berliner, the predator *Macrolophus Caliginosus*, and the parasitoid *T. achaeae* significantly reduced *T. absoluta* mines in tomato plants under greenhouse in Egypt (Kortam et al., 2014). Moreover, the combination of *B. thuringiensis* var. Kurstaki, *T. evanescens* Westwood, and pheromone mass trapping resulted in a significant reduction of *T. absoluta* larvae densities on tomatoes in open-field (Khidr et al., 2013). Furthermore, *Bacillus thuringiensis* and spinosad were efficient against *T. absoluta* larvae on tomato crops in open fields in Egypt (El-Aassar et al., 2015). Another study reported that some isolates of *Phthorimaea operculella* granulovirus were pathogenic to *T. absoluta* larvae under laboratory conditions (Ben Tiba et al., 2019). Also, EPNs were reported as promising against *T absoluta* in some studies (Batalla-Carrera et al., 2010; Garcia-del-Pino et al., 2013; Van Damme et al., 2016).

EPNs have some advantages comparing to the other biocontrol agents. Their management effect is fast only within 48 hours. Also, their ability to infect different insect species makes it suitable to control other tomato insect pests. Therefore, in this work, EPNs efficacy will be tested against *T. absoluta* larvae on tomatoes. EPNs are soil-inhabit animals that attack insect stages in soil. For foliar applications of EPNs, suitable formulations should be used against insect stages on foliage. Therefore, the aim of this work was to test a promising management system based on EPNs with an adjuvant against *T. absoluta*. Formulations of four nematode isolates for foliar application were tested against *T. absoluta* larvae on potted tomato plants under greenhouse conditions. The tested formulations were elaborated before through different bioassays. The experiment was conducted in the summer under simulated greenhouse conditions as they mostly appear in Egypt (e.g. no control of temperature or humidity). The aim is to test whether the good performance of the four isolates with the selected adjuvants under optimal conditions tests can be transferred to the challenging environment conditions.

VI.3. MATERIALS AND METHODS

VI.3.1. Infestation method

Thirty larvae of *T. absoluta* (2nd instar) were placed on the top side of the leaves on each tomato plant (eight leaves old). The larvae mined and fed on the leaves tissues at 25 °C. After one day, the larvae became 3rd instars, and the plants were used in the bioassay.

VI.3.2. The bioassay method

In this experiment, the efficacy of four nematode isolates *S. carpocapsae* BA2, *S. feltiae* Sf, *S. abbasi* abb, and *S. carpocapsae* J7 was evaluated against *T. absoluta* 3rd instar larvae on potted tomato plants under greenhouse conditions. Each isolate was applied in a volume of 250 ml on four potted tomato plants infested with *T. absoluta* 3rd instar larvae. The nematode suspensions were applied in a concentration of 5000 IJs/ml in 0.3% Nemaperfect® (e-nema GmbH). The applications were repeated after 24 h on the same plants. The applications were performed before the sunset. The suspensions were sprayed using a 500 ml hand-sprayer on four plants together till runoff. The plants in the untreated control received a solution of Nemaperfect® 0.3% instead of nematode suspensions. The pots were placed separately on a shelf in a complete randomized block design. Sticky glue was used to make borders at distances of 50 cm between the plants to avoid the escape of the larvae. The plants and the borders were checked after four days, and the dead and living larvae were counted. During the experiment period, the temperature range was 17-40 °C with an average of 27 °C, whereas the relative humidity ranged between 14 and 90% with an average of 51%. The dew point range was 3.2-19.5 °C, and the average was 14 °C. The entire experiment was performed three times at different time points during July 2018 in the greenhouses of JKI.

VI.3.3. Statistical analysis

The aim of the experiment was to compare the efficacy of the four nematode isolates applied against *T. absoluta* larvae on tomato plants under greenhouse conditions. Each isolate was applied on four tomato plants infested with about 30 larvae. The numbers of dead and living larvae per plant were counted, and larval mortality (%) was calculated. Each plant was considered as one replicate. The experiment was conducted three times at different time points. The nematode efficacy at different application times was analyzed. The statistical analysis and graphical presentation of results were performed using R software version “3.4.2” (R Core Team, 2017). Analysis of variance (ANOVA) was applied using function “aov”. Models were simplified and selected by using the Akaike's Information Criterion (AIC) and drop1 function with “F” test. The fitted model was with EPN isolate and trial as explanatory variables and larval mortality (%) as a response variable. Model suitability was visually checked by plotting the standardized residuals versus the fitted values, and dispersion was checked as well. The package ‘emmeans’ (Lenth, 2019) was used to calculate the estimated marginal means, posthoc tests at 0.05 significant level by the Tukey method, and 95% confidence intervals. The results were plotted using ‘ggplot2’ package (Wickham, 2016).

VI.4. RESULTS

All applied EPN isolates were able to infect *T. absoluta* larvae on tomato leaves inside or outside the galleries (Fig. VI.1), but with a different impact on target pest mortality. The nematode isolate *S. carpocapsae* BA2 recorded the highest mortality in *T. absoluta* larvae (85.54%), but it was not significantly different from the isolates *S. feltiae* Sf and *S. carpocapsae* J7 which resulted in 80.45, and 76.17%, respectively (Fig. VI.2). The nematodes of *S. abbasi* abb achieved 18.27% larval mortality, which was significantly lower than the other isolates.

The experiment was performed thrice under the greenhouse during the summer of 2018. The statistical fitted model was Model = aov (Mortality ~ Isolate + Trial). The model showed significant influences of both nematode isolates (df=3, F value=137.8, $p<0.0001$) and Trial (df=2, F value=3.7, p=0.032). However, applying the nematodes of *S. carpocapsae* BA2, *S. feltiae* Sf, or *S. carpocapsae* J7 at different time periods was not significantly different. The significant difference was noted only in the application of *S. abbasi* abb (Fig. VI.2). The highest recorded temperature during the application times was 40.1 °C and the average was 27 °C (Table VI.1). The applications were carried out before the sunset when temperatures gradually decreased. The nematode efficacy ranged between 83.67 and 87.59% for *S. carpocapsae* BA2, 78.06 and 82.17% for *S. feltiae* Sf, 70.50 and 80.31% for *S. carpocapsae* J7, and 6.30 and 30.75% for *S. abbasi* abb.

Figure VI.1: *Tuta absoluta* larvae infected with *Steinernema carpocapsae* BA2 applied in 5000 IJs/ml twice within 24 h on tomato plants in greenhouse.

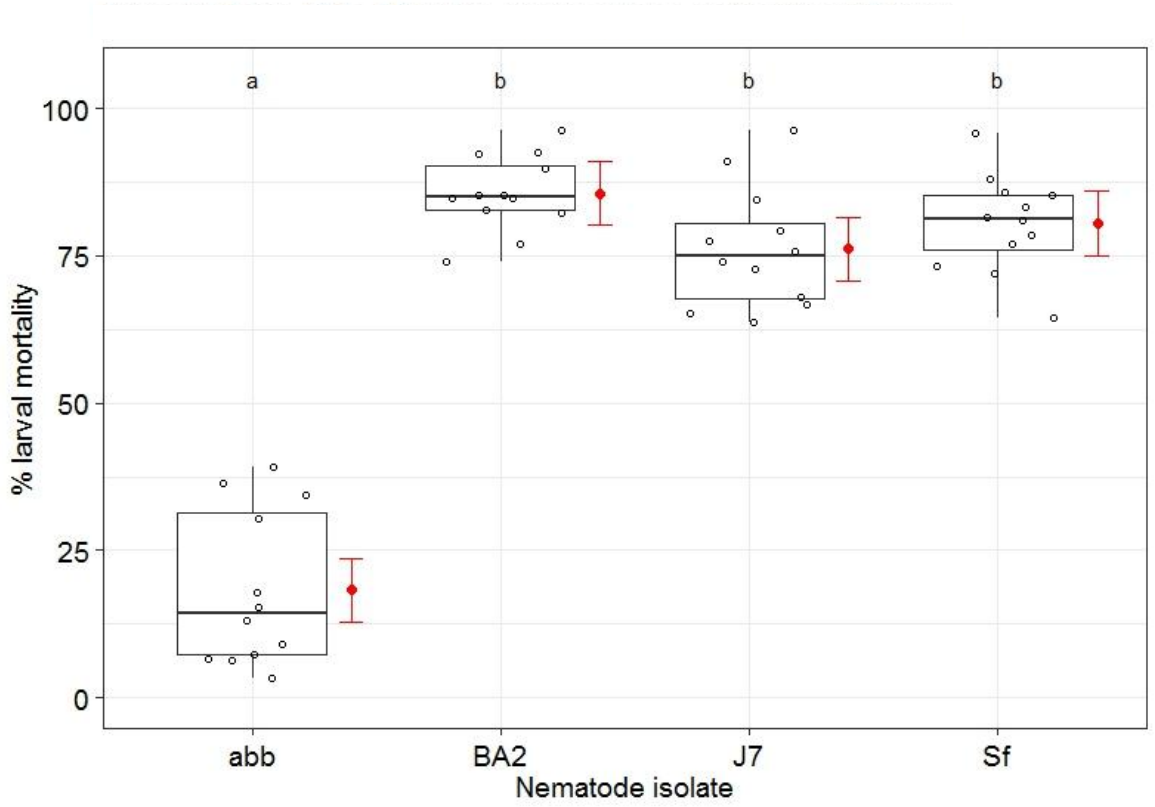

Figure VI.2: Larval mortality of *Tuta absoluta* in relation to treatment of four nematode isolates, *Steinernema abbasi* abb (abb), *S. carpocapsae* BA2 (BA2), *S. carpocapsae* J7 (J7), and *S. feltiae* (Sf), applied in 5000 IJs/ml twice within 24 hours. Boxplots are shown with individual observed values (n=12) as jittered points. The median is displayed by the horizontal line inside the boxplot. The estimated marginal means and 95% confidence intervals estimated from the fitted mixed model are displayed by the red color. The letters at the top indicate significant differences among the tested isolates according to posthoc tests ($p < 0.05$).

Table VI.1: The greenhouse conditions recorded during the experiment in July 2018 and the recorded larval mortalities (%) achieved by applications of four nematode isolates.

Time point	Conditions	Min.	Average	Max.	Larval mortality (%) achieved by nematode isolates			
					BA2	Sf	J7	abb
04.07.2018	Temperature (°C)	16.8	26.6	39.5	87.59	81.11	80.31	30.75
	Relative humidity (%)	13.3	51.5	93.3				
	Dew point (°C)	3.2	14.0	19.5				
17.07.2018	Temperature (°C)	17.8	26.1	38.1	85.37	82.17	70.50	6.30
	Relative humidity (%)	15.5	50.9	87.1				
	Dew point (°C)	7.3	13.2	19.5				
22.07.2018	Temperature (°C)	18.3	28.7	40.1	83.67	78.06	77.70	17.77
	Relative humidity (%)	18.4	46.6	87.0				
	Dew point (°C)	9.7	14.5	19.4				

VI.5. DISCUSSION

EPNs naturally occur in the soil and infect soil inhabiting insects. Therefore, EPNs are applied on soil applications against insect pests that spend part of their life cycle in soil. In some cases, EPNs were applied against insect pests in above-ground applications (Batalla-Carrera et al., 2010; Gözel and Kasap, 2015). The foliage application is very challenging, especially under high temperatures. However, the foliar application is recommended in some cases to protect the crop by targeting the most susceptible insect stage. In the current study, *T. absoluta* larvae are the economic stage and occur only on above-ground parts of tomato plants. Moreover, the larvae are the most susceptible stage to EPNs (Batalla-Carrera et al., 2010). For the previous reasons, the target stage was the 3rd instar larvae of *T. absoluta* in the current study. The tested EPNs were able to reach and infect *T. absoluta* larvae on tomato leaves even inside leaf galleries. The same results were reported by Batalla-Carrera et al. (2010), Van Damme et al. (2016), and Ndereyimana et al. (2019).

While the effectiveness of *S. abbasi* abb was surprisingly low, the three isolates of *S. carpocapsae* and *S. feltiae* resulted in high mortality levels in *T. absoluta* larvae in the present study. Various reports displayed high control rates of EPN species against *T. absoluta* (Batalla-Carrera et al., 2010; Garcia-del-Pino et al., 2013; Gözel and Kasap, 2015; Van Damme et al., 2016). In contrast, other reports displayed low efficacy levels of EPN species against *T. absoluta* larvae on tomato plants (Gözel and Kasap, 2015; Van Damme et al., 2016; Sabry et

al., 2016). In pot experiments, applications of *S. carpocapsae* and *S. feltiae* (1000 IJs/ml) twice within 24 hours resulted in high levels of efficacy (87 and 95%) against *T. absoluta* larvae on potted tomato plants (Batalla-Carrera et al., 2010). In open field trials, *S. feltiae* was the most efficient species with 90.7-94.3% mortality in *T. absoluta* larvae, whereas *S. affine* was the least efficient species with 39.3-43.7% larval mortality (Gözel and Kasap, 2015). Under greenhouse conditions, an application of *S. carpocapsae* All (250 IJs/ml) against *T. absoluta* larvae resulted in 12.9% of mine reduction (Sabry et al., 2016).

There are some challenges that may hamper the success of EPN foliar application. High temperatures and desiccation are the main factors that could limit EPNs control efficacy. Therefore, applying EPNs before sunset helps to avoid high temperature effects and fast desiccation. During the experiment time, the highest temperature degree was 40 °C and decreased gradually before sunset and at night. Some studies recommended applying the nematodes at dusk (Gözel and Kasap, 2015) or late afternoon (Mahmoud et al., 2016). Also, the use of a humectant adjuvant like Nemaperfect® with the application suspension could provide EPNs by moisture needed to reach the larvae of *T. absoluta* on leaves. In laboratory bioassays, two added adjuvants Addit® and Silwet L-77® increased the efficacy of the applied nematodes (Van Damme et al., 2016). The addition of surfactant-polymer adjuvant increased nematode mobility and larval mortality of the diamondback moth, *Plutella xylostella* (L.) (Schroer et al., 2005). Another study recommended using an adjuvant like Silwet L-77® or Penterra® (wetting agent) with EPNs application (Portman et al., 2016).

The application technique of EPNs is a crucial factor as well. The applied concentration levels are not fixed among the different studies. Also, the obtained results differ. In the current study, the application rate was 5000 IJs/ml ($5*10^9$ IJs/ha) applied twice within 24 hours (in total $10*10^9$ IJs/ha). The recommended application volume for insecticides is 400 litre/acre (10,000 liters/ha). According to commercial products of e-nema Company (Schwentinental, Germany), the recommended EPNs application rate is 50 IJs/cm^2 of soil surface ($5*10^9$ IJs/ha) against soil-borne insects. In pot experiment under greenhouse conditions, *S. carpocapsae* and *S. feltiae* in 1000 IJs/ml ($1*10^9$ IJs/ha) applied twice within 24 hours (in total $2*10^9$ IJs/ha) gained 87% and 94% mortality in *T. absoluta* larvae (Batalla-Carrera et al., 2010). In another pot experiment under greenhouse conditions, *S. carpocapsae* at a rate of 50 IJs/cm^2 ($5*10^9$/ha) resulted in a 50% reduction in *T. absoluta* (Kamali et al., 2018). The nematode efficacy was evaluated by determining the number of emerged *T. absoluta* adults per plant within 15 days after treatment (Kamali et al., 2018). An application of *S. carpocapsae* at a concentration of 250 IJs/ml ($250*10^6$ IJs/ha) reduced 12.9% of leaf mines produced by *T. absoluta* larvae on tomato plants under greenhouse conditions in Egypt (Sabry et al., 2016). In open field experiments in Turkey, application of *S. carpocapsae* and *S. feltiae* in a concentration of 50 IJs/cm^2 ($5*10^9$ IJs/ha) resulted in 49% and 94% mortality in *T. absoluta* larvae on tomato plants grown individually under cages (50 cm L * 50 cm W * 50 cm H), which were covered with organza (Gözel and Kasap, 2015).

In conclusion, the results demonstrate the potential of EPNs foliar application to control *T. absoluta* larvae on tomato crops under greenhouse conditions. The isolates of *S. carpocapsae* and *S. feltiae* resulted in high larval mortalities, and they were reliable in all three repetitions.

Chapter VII: General discussion

Many Egyptian farmers rely on tomato growing as their main income. Their joint efforts have also made Egypt one of the most important tomato-producing countries in the world. However, tomato growers face major challenges in crop management, especially due to the constant threat of various pests. Insects in particular directly damage various parts of the plant, such as leaves, stems, or fruits, or transmit diseases. There is always the risk of enormous crop losses, which is why farmers are more and more using chemical insecticides, with predictable negative effects on the environment and human health (Wood and Ehui, 2005; Van Bortel et al., 2008). In addition, sustainable crop protection in Egypt is also endangered by increasing insect resistance to many active ingredients of pesticides - and furthermore by the emergence of invasive pests such as the tomato leaf miner *Tuta absoluta* since the year 2009.

In view of this critical situation, the major goal of the research presented in this thesis was to elaborate a biological control method based on entomopathogenic nematodes (EPNs) as an effective and environmentally safe alternative for tomato cultivation in Egypt. Biological pest control has many positive features that make it a perfect option for efficient crop protection (Lacey et al., 2006). In many cases, it has achieved amazing success in agronomic practice and also opens up a thriving market for biocontrol suppliers and organically grown agricultural produce (van Lenteren et al., 2018). Among biological control agents, EPNs combine several advantages, making them first-choice candidates to solve the tomato pest problem in Egypt. In contrast to viruses or bacteria, they are not too host-specific and can attack a broader range of hosts, even belonging to different insect orders. This attribute is of great significance in the considered system, because several insect species belonging to Lepidoptera or Hemiptera (especially the whitefly *Bemisia tabaci*) occur in several generations and throughout the season on tomato in Egypt. These insects form the so-called Egyptian Tomato insect Pest Complex (ETPC) and the first challenge to solve in the thesis was to find a suitable EPN isolate, which can tackle all of them. As a result of this research, it was possible to compile a shortlist of four very potent EPNs (*S. abbasi* (abb), *S. carpocapsae* (BA2), *S. carpocapsae* (J7), and *S. feltiae* (Sf)) out of a collection of fifteen isolates by thorough screening at various dosages on the four lepidopteran target pests *Agrotis ipsilon*, *Helicoverpa armigera*, *Spodoptera littoralis*, and *T. absoluta*) in a standard sand bioassay. While many studies exist that have evaluated different isolates against individual host species (El Kifl, 1980; Tahir et al., 1995; Fetoh et al., 2009; Shamseldean et al., 2009; Batalla-Carrera et al., 2010; Garcia-del-Pino et al., 2013; Van Damme et al., 2016), the approach here was novel to select such isolates that are sufficiently effective against all relevant lepidopteran pests. Since these pests occur together on the above-ground parts of the tomato plant and feed there, the goal of optimal and cost-effective regulation can thus be realized much better. In addition, the grower does not have to carry out several treatments based on different biological control agents, as would be the case, for example, monophagous antagonists.

The whitefly *B. tabaci* is a plant sap-sucking insect and due to this different feeding behavior, another bioassay based on EPN-treated tomato leaves was necessary. Whiteflies are difficult targets for EPN compared to lepidopteran pests, as even high concentrations of IJs

resulted in relatively low mortality rates in other studies (Cuthbertson et al., 2003; Cuthbertson et al., 2007a,b). Nevertheless, the current study showed that the selected EPN isolates are able to invade this host. Especially high mortality rates of up to 80% were observed by application of the EPN isolate *S. feltiae* Sf against the immobile 2nd instar nymphs, indicating that this species is appropriate to combat this pest. The other EPN species also achieved mortality rates of over 50% when applied in high concentrations. These results proved that EPN species that are effective against lepidopterans could also successfully infest whiteflies. Thus, the first milestone of the work to find those EPNs that attack all targets of the ETPC was reached.

Another benefit of EPNs is the fact that they are able to kill their host very quickly (usually within 48 h), which is mainly an attribute of symbiotic bacteria (Griffin et al., 2005). In the present study, the EPN isolates that showed high virulence against all target hosts all belonged to the genus *Steinernema* (*S. carpocapsae, S. abbasi, S. riobrave, S. feltiae*), which is associated with bacterial strains of the genus *Xenorhabdus*. *Heterorhabditis* isolates carrying bacteria of the genus *Photorhabdus* also caused some mortality of ETPC targets, but not at the high level achieved by the *Steinernema-Xenorhabdus* combination. It was not within the scope of the present work to study in detail the mode of action on these host insects, but it certainly deserves further investigations to uncover the bacterial part (certain toxins, enzymes, etc.) in the successful breaking of resistance in these different host species. In general, the fast killing effect of EPN infection is a major advantage over many other biological control agents, such as koinobiont parasitoids, plant extracts, and slow-acting entomopathogens where the attacked hosts survive longer and even feed, causing further damage to the plant.

Nowadays, the invasive *T. absoluta* is the prevailing and most destructive pest on tomatoes in Egypt and was accordingly selected as the lead target pest to develop a control system against ETPC. Furthermore, the particular feeding behavior as a leafminer poses particular challenges to potential biocontrol agents. Antagonists need to reach the larvae inside their mines, where they are usually well protected against generalist predators or parasitoids, as well as against those chemical insecticides that do not penetrate the leaf surface. In addition, microbial pathogens need to be applied in a way that larvae get in contact with them or feed contaminated plant material. This is often not the case when larvae are already in their mines. In contrast, EPNs are mobile, perform an effective host searching behavior, also by following host cues/infochemicals (Griffin et al., 2005), and maybe small enough to enter the feeding mines of small *T. absoluta* larvae. The conducted leaf bioassay showed that all four “shortlist” EPN candidates were obviously attracted to mining *T. absoluta* larvae. They were able to infect the hosts in their galleries, thus confirming their efficiency against this pest under more natural conditions than it was tested before in the sand bioassay. Using the whole tomato leaflet instead of leaf disks as done in other studies (Van Damme et al., 2016) made the conditions even more challenging for EPNs to locate larvae by allowing the hosts to escape, as it probably also occur under real conditions. *Steinernema*-species are known to perform various host locations and mobility behaviors like “sit and wait”, cruising, or jumping on approaching insect larvae (Campos-Herrera, 2015) and this agility can also help them to cope with this particular host. In addition, the experiment has shown that all four “shortlist” EPN candidates are able to establish and/or move on the surface of the tomato leaf, an essential prerequisite for their use in this system. The isolate *S. carpocapsae* B2 was the most effective isolate, recording the lowest LC_{50} value against *T. absoluta* larvae in these bioassays.

Precautions must be taken to maintain EPN persistence on leaves long enough for the pests to be reached and infected. All insects of the ETPC stay on above-ground plant parts during their larval development; hence the EPNs have to be applied in the form of a foliar application. The next partial objective was therefore to achieve an optimization in the formulation of the EPN suspension. Rapid drying and inactivation of surface-applied EPNs are to be expected under field conditions in Egypt. Therefore, non-toxic components should, on the one hand, protect the EPNs, but on the other hand, also ensure good wetting of the hairy leaf surface and an even distribution of the EPNs in the suspension. Based on results from other studies, different adjuvants were selected and the effect on the application of *S. carpocapasae* B2 as the most effective isolate against *T. absoluta* on tomato plants was tested. Xanthan, Nemaperfect®, and Chitosan clearly improved the performance of the EPNs in these experiments and their use can be recommended. However, it needs to be checked if these substances are available and affordable for Egyptian farmers. The study also suggested a very simple method to increase the efficacy of the selected EPN isolates: the splitting of efficient dosages into half and applying the total EPN amount in two steps within 24 h also helped to increase the mortality of *T. absoluta* larvae significantly.

Certainly, the controlled laboratory conditions necessary for a standardized comparison of the effectiveness of the EPN isolates did not reflect the variable, often imponderable and challenging scenarios of Egyptian field cultivation of tomatoes. Even the conducted greenhouse trials with partly high temperatures and low humidity do not necessarily correspond to reality. Nevertheless, the results obtained allow developing the basis for an EPN based biocontrol system against the ETPC, which was also the declared aim of the research work and did not exist before. It was surprising that *S. abbasi*, which comes from a semi-arid region, performed worst in the greenhouse situation with high temperatures, while both *S. carpocapsae* isolates (including the German isolate, thus being from a temperate region) and *S. feltiae* produced satisfactory results. Further basic research could clarify the responsible traits of nematodes and their bacteria in order to find out possibilities to maintain or even manipulate them for increased effectiveness.

To conclude, for biocontrol of the ETPC, it can be recommended to apply effective EPN isolates in a double leaf application within 24 h in a formulation provided with adjuvants. As the target pests are mainly active during their larval development on the leaf surface at dusk or at night, an application at these times of day would be reasonable and better feasible for the farmers. Overall, this research has succeeded in establishing a basic system for an EPN-based control method.

The next steps would now be to have the three effective isolates (*S. carpocapsae* BA2 and J7, *S. feltiae* Sf) produced by a biocontrol producer, to find suitable and feasible application rates for field application, to test also EPN mixtures, and to make the system so cost-effective that Egyptian farmers adopt it and implement it into their pest management. If successful, the EPN-based biocontrol system against the ETPC would certainly have adaptive capabilities in other regions of the world where the aforementioned pests still make life difficult for farmers and prevent the harvest of healthy and tasty tomatoes.

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- Member of Safe Agriculture Group, Centre of Excellence for Advanced Sciences, National Research Centre. Dokki-Giza, Egypt. Nov. 2007- Apr. 2015.
- Agricultural Engineer, Salma Farm, Daltex Group. El-Bohyrah, Egypt. May-Nov. 2007.
- Agricultural Engineer, El-Tawheed for Agriculture Development Company. El-Bohyrah, Egypt. July 2006 - May 2007

SKILLS

Computer Skills:

- ICDL
- The R Software (statistical analysis)

Language Skills:

- Arabic: Native language
- English: very good
- German: very good

PUBLICATIONS

1. Saleh, M.M., Metwally, H.M., Abonaem, M., 2020. Commercialization of Biopesticides Based on Entomopathogenic Nematodes. In: Cottage Industry of Biocontrol Agents and Their Applications (pp. 253-275). Springer, Chambridge.
2. Abonaem, M., Herz, A., 2018. Developing foliar application of entomopathogenic nematodes for controlling *Tuta absoluta* on tomatoes. In: JKI (Hrsg.): 61. Deutsche Pflanzenschutztagung: Herausforderung Pflanzenschutz - Wege in die Zukunft ; 11. - 14. September 2018, Universität Hohenheim -Kurzfassungen der Vorträge und Poster. Julius-Kühn-Archiv 461, 482.
3. Abonaem, M., Herz, A., 2017. Comparative efficacy of four entomopathogenic nematode isolates against the tomato leafminer Tuta absoluta in laboratory leaf bioassay. In: JKI (ed.): 10th Young Scientists Meeting 2017, 8th -10th November in Siebeldingen - Abstracts. Berichte aus dem Julius Kühn-Institut 192, 22.
4. Hussein, M.A., Metwally, H.M., Abd Elraouf, M., 2015. Foliar application *of* native bio-formulated entomopathogenic nematodes against diamondback moth in aquaponic agriculture. Research Journal of Pharmaceutical, Biological and Chemical Sciences. 6, 1030-1035.
5. Abonaem, M.A.A. (2013). Proposed Measurements for the Efficiency of Entomopathogenic Nematodes. M.Sc. Thesis, Fac. Agri., Ain Shams Univ., Egypt, 86 pp.
6. Abdel Raouf, M., Kassab, A.S., Saleh, M.M.E., Hekal, A.M., Abbas, M.H., 2012. Image analysis as a new quality measurement of *Steinernema carpocapsae* (weiser) and *Heterorhabditis bacteriophora* poinar. Research Bulletin, Ain Shams Uni., 1-12.
7. Hussein, M.A., Abd El Aty, M.A., 2012. Formulation of two native entomopathogenic nematodes at room temperature. J. Biopest. 5, 23-27.

PRESENTATIONS

1. 36th Meeting DPG-DGaaE- Working Group „Nutzarthropoden & entomopathogene Nematoden“Nov. 27/28, 2018, Botanika, Bremen. Oral presentation.
2. ESN Conference, Sept. 9-13, 2018, in Ghent, Belgium. Oral presentation.
3. Young Scientist Meeting, Nov. 08-10, 2017 Julius Kühn-Institut, in Siebeldingen. Oral presentation.
4. 60th Deutsche Pflanzenschutztagung, Sept. 20-23, 2016 at the Martin-Luther University, Halle-Wittenberg. Oral presentation.
5. 34th Meeting of the Working Group on "Beneficial arthropods and Entomopathogenic Nematodes". Nov. 30 - Dez. 01, 2015 in Hannover. Poster.

Darmstadt, May 2021

Ehrenwörtliche Erklärung

Ich erkläre hiermit ehrenwörtlich, dass ich die vorliegende Arbeit entsprechend den Regeln guter wissenschaftlicher Praxis selbstständig und ohne unzulässige Hilfe Dritter angefertigt habe.

Sämtliche aus fremden Quellen direkt oder indirekt übernommenen Gedanken sowie sämtliche von Anderen direkt oder indirekt übernommenen Daten, Techniken und Materialien sind als solche kenntlich gemacht. Die Arbeit wurde bisher bei keiner anderen Hochschule zu Prüfungszwecken eingereicht.

Darmstadt, den ……

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