The potential of biopesticides for control of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae)

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“Success consists of going from failure to failure without losing enthusiasm”

-Winston Churchill

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"*All that we love deeply becomes a part of us*"- Helen Keller.

† Maria Jacoba Venter (1952–2018) and Johannes Lodewikus Venter (1949–2021) †

ABSTRACT

The invasive South American tomato pinworm, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) is one of the biggest threats to tomato production. Although other management strategies are used, control of this pest mainly relies on the application of synthetic chemical insecticides. Chemical control is, however, negatively affected by the cryptic feeding behaviour of the larvae and the pests' ability to rapidly evolve insecticide resistance. It is therefore crucial that the use of synthetic insecticides is integrated with more environmentally friendly management options. Biopesticides and pheromone-based control strategies represent some of the most promising alternatives. Firstly, this study aimed to determine the susceptibility of *T. absoluta* pupae to *Beauveria bassiana* and *Metarhizium anisopliae* administered as soil drench treatments and the possible effects of these entomopathogenic fungi (EPFs) on the fecundity and fertility of moths that emerged from pupae that were previously treated with EPFs. High pupal mortality was caused by the respective EPF products in bioassays and growth tunnel experiments. Fecundity of moths that emerged from surviving pupae, was significantly reduced compared to moths from untreated pupae. This provides evidence of the suppressive potential of EPF conidia administered as a soil drench for control of *T. absoluta* pupae. Secondly, this study aimed to determine the genetic diversity of South African *T. absoluta* populations, and whether moths were able to reproduce parthenogenetically. The strain composition and frequency of *Wolbachia* infection in *T. absoluta* populations were also investigated. The genetic diversity of *T. absoluta* in South Africa is low, and consists of only four haplotypes. A novel haplotype was reported from South Africa during this study. Despite high *Wolbachia* infection rates (≥ 95%) of virgin *T. absoluta* females with a single *Wolbachia* strain (ST41), no parthenogenetic reproduction occurred. Control failure of pheromone-based strategies in South Africa can therefore not be attributed to *Wolbachia*-induced parthenogenesis. Lastly, this study aimed to determine the systemic efficacy of spinosad applied as a soil drench for control of *T. absoluta,* the persistence of spinosad in the leaves and fruit, and the potential effect of this drench application on tomato plant growth parameters. Although spinosad concentration in leaves decreased gradually over time, high larval mortalities (> 96%) were recorded for the duration of the experiment, which lasted 25 days. The spinosad residues detected in fruit were well below the maximum residual limits mandated for South Africa. This study demonstrated the systemic properties of spinosad for control *T. absoluta* in tomato. Future research should investigate the compatibility of spinosad and entomopathogenic fungi applied as a soil drench, taking into account the possibility of resistance evolution, while also incorporating pheromone-based strategies as more environmental friendly additions to IPM programmes.

Key words: biopesticides, entomopathogenic fungi, parthenogenesis, spinosad, tomato pinworm

PREFACE

This thesis follows the article style format. In accordance with the prescriptions of the North-West University (NWU), the articles appear in published format and manuscripts were prepared following the instructions to authors of internationally accredited, scientific journals (Table A). Chapters that are not intended to be published were prepared following the general guidelines for theses and dissertations set out by the NWU.

Table A. Publication status of articles contained in thesis.

Chapter	Status	Journal
Chapter 1	-	NWU general guidelines
Chapter 2	Published	*Insects* (MDPI)
Chapter 3	Submitted	*Entomologia Generalis.*
Chapter 4	Prepared for submission	*Journal of Economic Entomology*
Chapter 5	-	NWU general guidelines

The instructions to authors of the journals (submitted chapters) are included in Appendix A-C. Chapter 1 and 5 were adjusted according to a derivative of the NWU general guidelines. Appendix E contains the declaration that the work presented within this thesis has been language edited.

Table B details the contributions of authors for each article/manuscript and provides consent for use as part of this thesis.

Author	Article	Contribution	Consent
R Erasmus	1 - 3	Principle investigator: responsible for study conceptualization, study design, execution of experiments, data analysis, data interpretation and writing of manuscripts. Served as first author.	
M.J. du Plessis	1 - 3	As promoter, contributed to the conceptualization of the study and supervised the execution of experiments. Also provided guidance and intellectual input on data analysis and writing of manuscripts.	M. J. du Plessis.
J. van den Berg	1 - 3	As co-promoter, contributed to the conceptualization of the study and supervised the execution of experiments. Also provided guidance and intellectual input on data analysis and writing of manuscripts.	J van den Berg
P. Jansen van Rensburg	3	As analytical specialist, responsible for implementing a pre/selected LC-MS/MS method, setting up and performing extraction, data acquisition and quantitative data analysis.	

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CHAPTER 1

Introduction, literature review and thesis structure

1. Introduction

Invasive species represent a major threat to both managed and natural systems (Asplen *et al.*, 2015; Clavero & García-Berthou, 2005; Pimentel *et al.*, 2000). Economic trade and the introduction of invasive species have been interconnected since the dawn of the Industrial Revolution (Kolar & Lodge, 2001). However, since the advent of globalization, the number and diversity of biological invasions have increased drastically (Essl *et al.*, 2011; Hulme, 2009). Among invasive alien species, newly established insect pests can increase control costs, reduce yields, and result in the overreliance on synthetic insecticides, which in turn leads to the disruption of integrated pest management (IPM) programs (Pimentel *et al.*, 2000; Ragsdale *et al.*, 2011). IPM enables the successful control of insect pests while causing limited harm to the environment by following a science-based decision-making process (Kogan, 1998).

Tomato, *Solanum lycopersicum* L. (Solanaceae), is one of the most widely cultivated horticultural crops, second only to potato (Biondi *et al.*, 2018; El-Shafie, 2020). Approximately 5 million hectares are planted with tomato worldwide, producing 180 million tons per year worth 87.9 billion US$ in 2016 (El-Shafie, 2020). In sub-Saharan Africa, tomato is one of the major horticultural crops that small-scale farmers rely on for both income and nutrition (Zekeya *et al.*, 2017). However, tomato production is constrained by several factors, including arthropod pests (Guimapi *et al.*, 2016). The most important species in the tomato pest complex includes the following: the African bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), red spider mite, *Tetranychus evansi* (Baker and Pritchard) (Acarida: Tetranychidae), leafminers, *Liriomyza* spp. (Mik) (Diptera: Agromyzidae) and thrips, *Frankliniella* spp. (Karny) (Thysanoptera: Thripidae), in addition to the invasive South American tomato pinworm, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae)

(Campos *et al.*, 2017; Desneux *et al.*, 2021; Guedes & Picanço, 2012), which has invaded more than 90 countries outside its native South America (EPPO, 2021; Santana *et al.*, 2019) and threatens approximately 87% of worldwide tomato production (Campos *et al.*, 2017; Desneux *et al.*, 2011).

1.1 Origin and distribution of *Tuta absoluta*

Although native to Peru (Vargas, 1970), the tomato leaf miner is widespread through most South American countries where it has been recognized as a pest of tomato for over 50 years (Desneux *et al.*, 2010; Guedes & Picanço, 2012). The pest only occurred throughout South American countries until 2006, before it was reported for the first time in the eastern parts of Spain (Desneux *et al.*, 2010; Urbaneja *et al.*, 2007). Since then, *T. absoluta* has extended its range to various other European countries, including France (2008), Italy (2008), Greece (2009), Malta (2009), Bulgaria (2009), Portugal (2009), Netherlands (2009), United Kingdom (2009) and Serbia (2011) (Dandria & Catania, 2009; EPPO, 2009a, 2009c, 2009d, 2009e; Harizanova *et al.*, 2009; Sannino & Espinosa, 2009; Tonnang *et al.*, 2015; Toševski *et al.*, 2011).

The pest finally reached the African continent during 2008 where it was identified in Morocco and Algeria (Brévault *et al.*, 2014). *Tuta absoluta* continued its dispersal through the African continent with reports from Egypt (2010), Sudan and South Sudan (2011), Ethiopia (2012), Kenya (2013), Tanzania (2014) and Senegal (2014) (Biondi *et al.*, 2015; Brévault *et al.*, 2014; Goftishu *et al.*, 2014; Mohamed *et al.*, 2015; Moussa *et al.*, 2013; Pfeiffer *et al.*, 2013; Tonnang *et al.*, 2015). During 2016 the South African government released the first pest alert for the detection of *T. absoluta* in the eastern parts of the Mpumalanga province (DAFF, 2017b).

The distribution of *T. absoluta* in Africa (Fig. 1.1) indicates that this pest is able to invade and spread in most areas of the continent partly due to the similar climatic and ecological conditions to those in its native range (Tonnang *et al.*, 2015). Although the spread of *T. absoluta* via flight (active) and wind (passive) is possible, human activities such as intensive cultivation and cross border trade of tomato fruit plays a more significant role in this pest’s ability to invade new areas (Desneux *et al.*, 2011; Tonnang *et al.*, 2015).

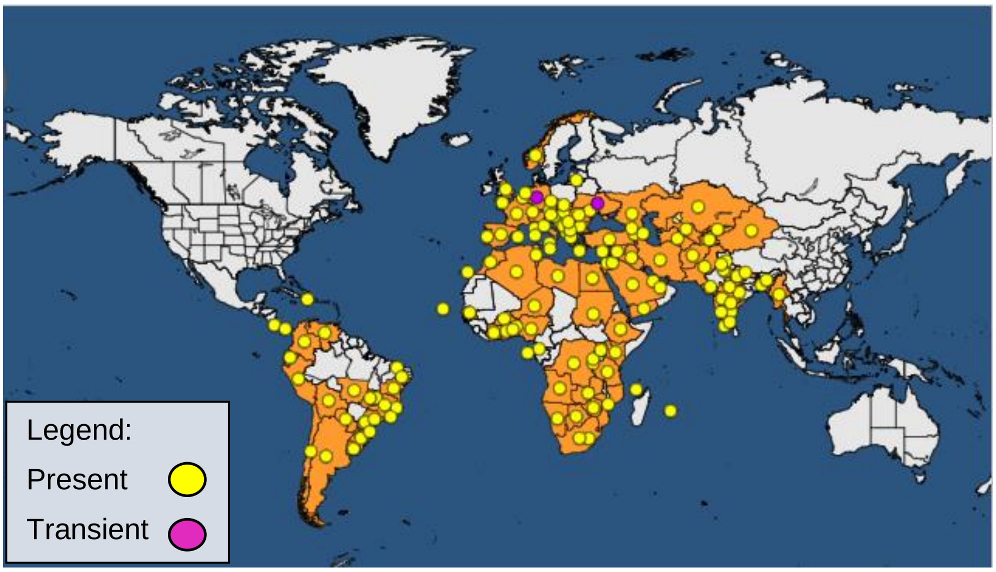

Figure 1.1 Geographical distribution of *Tuta absoluta* (From EPPO, 2021).

1.2 Biology and behaviour of *Tuta absoluta*

The life cycle of *T absoluta* is comprised of four biological stages: egg, larva, pupa and moth (adult) (Desneux *et al.*, 2010; Harbi *et al.*, 2012). The development rate of *T. absoluta* depends on environmental conditions and its life cycle can be completed in 23.8 days at 27.1 ˚C (Barrientos *et al.*, 1998; Nayana & Kalleshwaraswamy, 2015). Due to its multivoltine reproduction (Erdogan & Babaroglu, 2014; Pereyra & Sánchez, 2006), *T. absoluta* has the ability to produce between 10-13 generations per year, resulting in rapid population increases, provided the climatic conditions are favourable and the availability of food sources (Desneux *et al.*, 2010; Zekeya *et al.*, 2017).

Gravid female moths can deposit up to 260 eggs on leaves, stems and to a lesser extent on the fruit of tomato plants (Uchoa-Fernandes *et al.*, 1995). Larvae hatch from eggs after 5 days, after which they penetrate and feed on leaf, stem and fruit mesophyll tissue to complete four larval instars in approximately 9-14 days (Bawin *et al.*, 2015; Nayana & Kalleshwaraswamy, 2015; Pfeiffer *et al.*, 2013). The mining of the aerial mesophyll tissue in leaves results in damage symptoms which are commonly referred to as galleries (Erdogan & Babaroglu, 2014). Fourth-instar larvae normally pupate in dry leaf galleries (Pfeiffer *et al.*, 2013; Uchoa-Fernandes *et al.*, 1995) or in the soil for approximately 7 to 10 days (at 27˚C) before moths emerge (Bajracharya & Bhat, 2018; Nayana & Kalleshwaraswamy, 2015).

Although *T. absoluta* prefers tomato as its primary host plant, it can successfully reproduce and develop on several other Solanaceae such as potato (*Solanum tuberosum* L.), pepper (*Capsicum annuum* L.), sweet pepper (*S. muricatum* L.), tobacco (*Nicotiana tabacum* L.) and eggplant (*S. melongena* L.) (Galarza, 1984; Mohamed *et al.*, 2015; Pereyra & Sánchez, 2006; Portakaldali *et al.*, 2013). Suitable non-crop solanaceous host plants include weeds such as nightshade (*S. nigrum* L.), sticky nightshade (*S. sisymbriifolium* Lam.) Jimson weed (*Datura stramonium* L.), long spined thorn apple (*D. ferox* L.) bindweed (*Convolvulus arvensis* L.), lambs-quarters (*Chenopodium album* L.), *S. bonariense* L. and *Lycopersicum puberulum* Phil. (Mohamed *et al.*, 2015; Portakaldali *et al.*, 2013). Since the pests' arrival in Europe, species belonging to the Fabaceae family were also reported as host plants, for example, common bean (*Phaseolus vulgaris* L.) in Italy, cowpea (*Vigna unguiculata* L. Walp.), broad bean (*Vicia faba* L. Walp.) and alfalfa (*Medicago sativa L.*) in Iraq and a wild radish species (*Raphanus raphanistrrum* L.) as a member of the Brassicaceae family (Abdul-Rassoul, 2014; Abdul-Ridha *et al.*, 2012; EPPO, 2009b; Harizanova *et al.*, 2009; Mohamed *et al.*, 2015).

1.3 Economic importance of *Tuta absoluta*

In South America, the tomato pinworm is a devastating pest of tomato in both field and greenhouse cultivation (Barrientos *et al.*, 1998; Estay, 2000), where it attacks all developmental stages of plants (Cifuentes *et al.*, 2011). Feeding damage is caused by all larval instars throughout the tomato crop cycle (Harizanova *et al.*, 2009). Females oviposit preferentially on leaves and to a lesser extent on leaf veins, stems and fruit (Estay, 2000). Newly hatched larvae penetrate and feed on mesophyll tissue of all aerial parts of plants. Damage caused to tomato leaves as a result of feeding on mesophyll negatively affects the plant's photosynthetic capacity leading to lower tomato yields (Bawin *et al.*, 2015; Desneux *et al.*, 2010) (Fig 1.2 A). Galleries in stems and growth tips generally halt plant development and could lead to necrosis

(Gebremariamd, 2015). However, the most economically devastating damage is to the fruit, where larval galleries in fruit are accompanied with secondary pathogens leading to fruit rot (Cifuentes *et al.*, 2011; Desneux *et al.*, 2010) (Fig 1.2 B).

The different damage symptoms caused by *T. absoluta* result in a significant reduction in both yield and fruit quality. Without appropriate control, *T. absoluta* can cause yield losses in tomato ranging between 80-100% (Apablaza, 1992; Desneux *et al.*, 2010). This high pest status is concerning, especially against the background of the high nutritive value of tomato and the role it plays in small-scale trade in sub-Saharan Africa (Brévault *et al.*, 2014). For example, in Tanzania, tomato yield decreased to such an extent that farmers do not want to cultivate the crop anymore due to the costs and losses associated with *T. absoluta* damage (Zekeya *et al.*, 2017). The economic impact of *T. absoluta* is reflected in the increasing costs of tomato production, which include a decrease in marketable products, potential loss of trading partners due to export restrictions and additional costs of pest management strategies (Campos *et al.*, 2017).

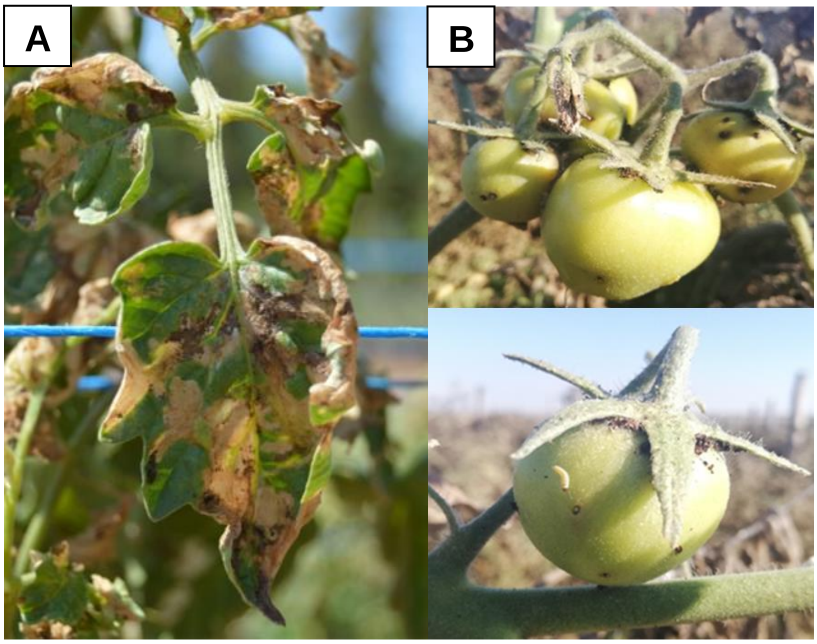

Figure 1.2 Damage symptoms of *Tuta absoluta* in (A) leaves and (B) developing fruit. (Photos: Reynardt Erasmus).

1.4 Methods to control *Tuta absoluta*

Prophylaxis is one of the easiest and also most inexpensive ways to control a pest infestation (Berlinger *et al.*, 1999). Accordingly, the best strategies to control *T. absoluta* focus on the prevention of pest outbreaks, which include the integrated application of cultural, biotechnological, biological and chemical control measures (Biondi *et al.*, 2018; Desneux *et al.*, 2021; El-Arnaouty *et al.*, 2014; Monserrat Delgado, 2010; Zekeya *et al.*, 2017). The use of synthetic insecticides is only recommended when preventative strategies are insufficient to maintain a pest population below economic threshold levels (Urbaneja *et al.*, 2013).

1.4.1 Cultural practices

Cultural control plays a significant role in making the environment unfavourable for pest insects to survive in (Kfir *et al.*, 2002). Most cultural practices recommended for the control of *T. absoluta* aim to prevent initial infestation. Crop isolation is one of the most accepted cultural control strategies to restrict flying pests such at *T. absoluta* (Retta & Berhe, 2015). This can be achieved in greenhouses by screening vents, installing double-doors and ensuring that all netting has a maximum density of 9 x 6 threads/cm^2 (Abbes *et al.*, 2012; Biondi *et al.*, 2018; Desneux *et al.*, 2010; Monserrat Delgado, 2008). However, screening of greenhouses may reduce colonization of natural beneficial agents as well as ventilation in greenhouses (Illakwahhi & Srivastava, 2017; Monserrat Delgado, 2008). The management of plant material also forms an integral part of preventative cultural control against *T. absoluta*, which involves the transplant of only pest free seedlings, removal of damaged plant material at low infestation densities (Abbes *et al.*, 2012) and the control of weeds that can possibly serve as an alternative host plants for the pest (Abbes *et al.*, 2012; Desneux *et al.*, 2021). Additionally, crop residues must be removed as soon as possible by burying residues or by covering them with transparent plastic film to allow fermentation (Mansour *et al.*, 2018; Tapia *et al.*, 2010).

Crop rotation with non-solanaceous crops is vital to break the pest’s life-cycle in an effort to reduce population build-up (Desneux *et al.*, 2021). In areas where farms specialize in tomato production only, greenhouses should be emptied and sealed for

4-8 weeks to rid the environment of this pest (Monserrat Delgado, 2009, 2010). Remaining adults emerging from the soil will die during this period.

1.4.2 Pheromone-based strategies

Sex pheromones are chemical cues released by an organism to attract the opposite sex of the same species for mating (Caparros Megido *et al.*, 2013). In the case of lepidopterans, mate location predominantly involves the upwind flight of males towards a chemical attractant released by females (Cardé & Minks, 1995). Female sex pheromones, which usually consists of two or more compounds, not only evokes long distance male attraction but also prompts courtship behaviour (Linn *et al.*, 1987). Due to their integral role in insect mating behaviour, the use of sex pheromones forms a major part in the management of lepidopteran pests (Desneux *et al.*, 2021). Pheromone-based control strategies are non-toxic, species specific, safe for workers and not harmful to beneficial insects, thus forming the basis for sustainable and environmentally friendly integrated pest management strategies (Witzgall *et al.*, 2010).

Svatoš *et al.* (1996) identified the female-emitted sex pheromone of *T. absoluta* as a 90:10 mixture of (3E, 8Z, 11Z)-tetradecatrien-1-yl acetate and (3E, 8Z)-tetradecadien-1-yl acetate. Since then, their synthesis has been improved, resulting in higher selectivity and yields (Puigmartí *et al.*, 2015). The most successful and widespread application of sex pheromones is in the deployment of baited traps to monitor initial pest presence and population densities (Harizanova *et al.*, 2009; Witzgall *et al.*, 2010). These pheromone traps serve as a precursor, which enables a farmer to detect the presence or absence of a pest within a crop (Caparros Megido *et al.*, 2013). After initial pest detection, baited pheromone traps are principally used to monitor population levels and initiate the introduction of control methods such as the application of synthetic insecticides once action thresholds are reached (Salas, 2004). In South America, pheromone traps are used for monitoring in combination with economic injury levels, which were established at 45 ± 19.5 (mean ± SE) males/trap/day (Benvenga *et al.*, 2007). Although definitive trap densities have not been established for *T. absoluta* monitoring systems, an initial reliable monitoring program could be achieved with as little as 1 – 4 traps/ha (Mansour *et al.*, 2019).

According to Monserrat Delgado (2008), the number of males caught per pheromone trap indicates the risk of infestation and is considered as low risk for less than 3 moths/trap/week, moderate for 4 – 30 moths/trap/week and high for more than 30 moths/trap/week.

Although used as an effective monitoring tool, synthetic pheromones can also be applied to effectively control insect populations through mating disruption and mass trapping (Witzgall *et al.*, 2010). Mating disruption relies on the saturation of the atmosphere with synthetic pheromones, causing disorientation and communication interference (Cocco *et al.*, 2013; Witzgall *et al.*, 2010). This influences the ability of males to locate females for mating, leading to a reduction in population density in subsequent generations (Cardé & Minks, 1995; Cocco *et al.*, 2013; Witzgall *et al.*, 2010). For this strategy to be effective, large amounts of pheromones need to be deployed at rates of 500 – 1000 traps/ha (Cocco *et al.*, 2013; Vacas *et al.*, 2011). Research on the effectiveness of mating disruption in open field and greenhouse tomatoes have however produced mixed results (Michereff-Filho *et al.*, 2000; Vacas *et al.*, 2011). The high levels of interruption in *T. absoluta* male orientation (60 – 90%) in small plots in Brazil did not result in a reduction in leaflet and fruit damage (Michereff-Filho *et al.*, 2000). However, Vacas *et al.* (2011) reported effective control of *T. absoluta* when using mating disruption in high-containment greenhouses, but not in low-containment greenhouses.

Mass trapping involves the deployment of high quantities of pheromone lures in combination with large insect retention traps at strategic positions over a target area (Jones, 1998; Witzgall *et al.*, 2010). The mass trapping strategy aims to trap male *T. absoluta* moths in numbers high enough to decrease mating events, resulting in less crop damage (Jones, 1998; Witzgall *et al.*, 2010). In the case of *T. absoluta*, water traps are most commonly used in mass trapping strategies (Desneux *et al.*, 2021). This type of trap is easy to maintain and has a large trap capacity (Salas, 2004). Pheromone-baited trap densities for mass trapping have been established as 40 – 50 traps/ha in open field and 20 – 25 traps/ha inside greenhouses (Bolckmans, 2009). Since *T. absoluta* males may mate up to 6 times, even the presence of only a small number of males may result in high numbers of offspring (Silva, 2008; Wang *et* *al.*, 2021). Therefore, a large portion of males must be trapped before a population could be suppressed by the mass trapping method.

Both mass trapping and mating disruption are rarely sufficient to control *T. absoluta* populations on their own and should preferably be used in combination with other control methods as part of an integrated approach (Cherif *et al.*, 2017; Desneux *et al.*, 2021). Several factors could result in failure of these pheromone based strategies, including the migration of mated females into a treated area, a high pest population density, and reproductive behaviour that counters the efficacy of these control methods (Caparros Megido *et al.*, 2012; Michereff-Filho *et al.*, 2000). Amongst these counteracting reproductive behaviours, polygyny in males and polyandry in females, the ability of *T. absoluta* to reproduce asexually through parthenogenesis has also been linked to failure of pheromone-based control strategies (Abbes & Chermiti, 2014; Caparros Megido *et al.*, 2012; Desneux *et al.*, 2021; Wang *et al.*, 2021).

1.4.2.1 Parthenogenesis in *Tuta absoluta*

Parthenogenesis is a natural form of asexual reproduction in which growth and development of embryos occur without fertilization by sperm (Mittwoch, 1978). Parthenogenesis is a relatively rare form of reproduction among lepidopterans (Lynch, 1984; Suomalainen, 1962). Thelytokous parthenogenesis, which results in only female progeny, is however found in several species of psychids (Lepidoptera: Psychidae) (Lynch, 1984). In other families of Lepidoptera this form of asexual reproduction is the exception and is termed as tycho- or accidental parthenogenesis (Ball, 2001). Approximately only 20 other lepidopteran species belonging to several families, *viz*. Bombycidae, Gelechiidae, Gracillariidae, Lasiocampidae, Liparidae, Nepticulidae, Saturnidae, Sesiidae, Sphingidae, are known to be able to reproduce through deuterotokous parthenogenesis (producing progeny of equal sex ratio) (Gangopadhyay *et al.*, 2005; Liu *et al.*, 2018; Menken & Wiebosch-Steeman, 1988; Mozŭraitis *et al.*, 2002; Taylor, 1954).

The ability of *T. absoluta* to undergo deuterotokous parthenogenesis was first reported in 2012 in a population collected from Saint Andiol, France (Caparros

Megido *et al.*, 2012). This phenomenon was later confirmed in Tunisian and Chinese *T. absoluta* populations (Abbes & Chermiti, 2014; Wang *et al.*, 2021). Caparros Megido *et al.* (2012) reported that 20% of isolated virgin females were able to lay viable eggs, with a hatch rate of 39.9% of unfertilized eggs. A study by Abbes and Chermiti (2014) reported parthenogenesis in all three Tunisian populations tested, with an average of 31% of isolated females being able to produce viable eggs with hatch rates ranging between 11.36 and 17.02%. In the reported case of parthenogenesis in a Chinese population, only 1.1% of unfertilized eggs hatched (Wang *et al.*, 2021). All three accounts of parthenogenesis in *T. absoluta* reported the following similar observations, *viz*. that isolated virgin females produced fewer eggs, these eggs had lower hatch rates and the females survived longer when compared to mated females (Abbes & Chermiti, 2014; Caparros Megido *et al.*, 2012; Wang *et al.*, 2021). Even though sexual reproduction leads to better overall fitness and more offspring, the adaptive characteristic to produce viable eggs in the absence of males could improve the potential of this pest to spread and establish in newly invaded areas (Wang *et al.*, 2021). Some questions remain about the origin and cause of parthenogenesis in *T. absoluta*. Whether it's an adaptive reproductive behaviour, purely accidental, or the result of manipulation by the endosymbiont *Wolbachia*, parthenogenesis under field conditions could counteract the efficiency of pheromone-based strategies (Abbes & Chermiti, 2014; Caparros Megido *et al.*, 2012).

1.4.2.2 *Wolbachia*: master manipulator

Wolbachia is a group of endosymbiotic bacteria, maternally transmitted through the egg cytoplasm to offspring (Škaljac *et al.*, 2012; Werren & Windsor, 2000; Werren *et al.*, 2008). Until the late 1990's, *Wolbachia* were believed to be an unimportant rare genus of bacteria (Werren *et al.*, 2008). However, since the development of molecular typing techniques, *Wolbachia* were found to be prevalent in insects and other arthropods such as isopods, spiders, scorpions and mites (Werren *et al.*, 2008). A meta-analysis of 20 different studies examining the frequency of *Wolbachia* infections in insects estimated that more than 65% of insect species harbour *Wolbachia* (Hilgenboecker *et al.*, 2008), including *T. absoluta* (Carvalho *et al.*, 2018; Škaljac *et al.*, 2012). *Wolbachia*'s pandemic distribution can be attributed to several strategies that ensure the vertical transmission to host offspring through the manipulation of the reproductive system of the arthropod host (Saridaki & Bourtzis, 2016). These strategies include cytoplasmic incompatibility (CI), male killing, feminization of genetic males, and parthenogenesis (Breeuwer *et al.*, 1992; Hurst *et al.*, 1999; Saridaki & Bourtzis, 2016; Stouthamer *et al.*, 1993; Werren, 1997; Werren *et al.*, 2008).

The *Wolbachia*-induced feminization phenotype was first described in isopods (Rousset *et al.*, 1992) and later in two species of insects, namely *Eurema hecabe* (L.) (Lepidoptera: Pieridae) and *Zyginidia pullula* (Boheman) (Hemiptera: Cicadellidae) (Hiroki *et al.*, 2002; Negri *et al.*, 2006). Although the exact mechanism of this phenotype is unclear, *Wolbachia* seems to continuously interfere with the sex-determination pathway throughout development, turning genetic males into females (Rigaud, 1997). *Wolbachia*-induced male killing has been described in three insect orders, *viz*. Lepidoptera, Coleoptera and Diptera (Fialho & Stevens, 2000; Fujii *et al.*, 2001; Hurst *et al.*, 2000). In the absence of *Wolbachia*, genetic female progeny dies during larval development, while in the presence of *Wolbachia*, genetic males die during larval development through lethal feminization (Werren *et al.*, 2008). Benefits of male killing might include more food for surviving female progeny, reduced intensity of antagonistic interactions between siblings and a reduced chance of inbreeding (Charlat *et al.*, 2003).

The most frequently observed and studied *Wolbachia*-induced phenotype is CI, which has been reported in several insect orders (Werren *et al.*, 2008). Cytoplasmic incompatibility is a form of conditional sterility whereby the fertilized eggs of females not infected with the same strain of *Wolbachia* as the males with which they mated, will undergo embryonic death (Hurst & Frost, 2015; Sinkins, 2004; Werren *et al.*, 2008). Despite considerable research efforts on the effect and proposed mechanisms, the exact mechanism of CI remains elusive (Poinsot *et al.*, 2003; Saridaki & Bourtzis, 2016). It is however clear that the incompatibility phenotype is associated with asynchrony in the development of female and male pronuclei (Serbus *et al.*, 2008) and that CI produces a delay in the recruitment of the H3.3/H4 histone-complex to the male pronucleus (Landmann *et al.*, 2009).

As in CI, *Wolbachia*-induced parthenogenesis is the result of a disruption of the cell cycle during the early embryonic development (Werren *et al.*, 2008). *Wolbachia*-induced parthenogenesis has only been documented in arthropod species with arrhenotokous development such as hymenopterans, thrips and mites (Arakaki *et al.*, 2001; Huigens & Stouthamer, 2003; Kumm & Moritz, 2008; Reumer *et al.*, 2010; Stouthamer *et al.*, 1990; Weeks & Breeuwer, 2001). Instead of males developing from unfertilized eggs (arrhenotoky), *Wolbachia*-infected females produce only female progeny which are able to transmit the bacteria to their offspring, unlike male progeny (Werren, 1997; Werren *et al.*, 2008). Although it is less common than CI and has only been documented in haplodiploid species, the *Wolbachia*-induced parthenogenesis phenotype has often been suggested to be the cause of parthenogenesis in *T. absoluta* (Carvalho *et al.*, 2018).

1.4.3 Chemical control

1.4.3.1 Efficacy of insecticides

Despite the search for alternative methods to control *T. absoluta*, farmers still mainly rely on the application of chemical insecticides, especially when invasive species such as *T. absoluta* are the target pests (Biondi *et al.*, 2018; Erasmus *et al.*, 2021; Guedes *et al.*, 2019). However, insecticide applications are often not as effective as expected in controlling *T. absoluta*, partly due to the endophytic feeding habit of larvae (Alsaedi *et al.*, 2017; Cocco *et al.*, 2013). Insecticide efficacy usually decreases if an insect population is well established prior to the reproductive growth stage of tomato plants (Guedes *et al.*, 1994; Picanço *et al.*, 1995). Furthermore, the pests' high reproductive potential accompanied with intensive use of insecticides has led to resistance development against a wide range of chemical insecticides, including abamectin, cartap hydrochloride, chlorantraniliprole*,* lambda-cyhalothrin, flubendiamide, methamidophos, permethrin, spinosad and tau-fluvalinate (Campos *et al.*, 2014; Haddi *et al.*, 2012; Roditakis *et al.*, 2015; Siqueira *et al.*, 2000; Siqueira *et al.*, 2001).

Despite the limited success of chemical control, *T. absoluta* detection in newly invaded areas is usually characterized by a large increase in insecticide application.

Guedes and Siqueira (2012) reported that the average number of sprays required for pest control per tomato cultivation cycle increased from 10 – 12 sprays to more than 30 sprays after the detection of *T. absoluta* in Brazil. Insecticide resistance often leads to more frequent applications of insecticides at higher dosage rates and thus, an increase in selection pressure that accelerates the rate of pest resistance evolution as well as environmental pollution (Barati *et al.*, 2018).

Insecticides registered for the control of *T. absoluta* globally, belong to 12 Insecticide Resistance Action Committee (IRAC) mode of action (MoA) groups, with the majority of them inhibiting growth of the insect or affecting the insect's nervous system (IRAC, 2017). In South Africa, a limited number of synthetic insecticidal products are registered for the control of *T. absoluta* (Table 1.1). These products comprise eight registered active ingredients belonging to seven IRAC MoA groups (DAFF, 2017a).

Table 1.1 List of insecticides registered in South Africa for the control of *Tuta absoluta* (DAFF, 2017a).

Active ingredients	**Crop**	**IRAC MoA group**
Spinosad	Tomato, cabbage, cucurbits, lettuce, potato and spinach	5
Spinetoram	Tomato and potato	5
Emamectin benzoate & lufenuron	Tomato, capsicums, eggplant and tobacco	6 &15
Indoxacarb	Tomato, eggplant and peppers	22
Chlorantraniliprole	Tomato, eggplant, potato and tobacco	28
Chlorantraniliprole & lambda-cyhalothrin	Tomato, capsicums, eggplant, potato and tobacco	28 & 3
Flubendiamide	Tomato, potato and tobacco	28

1.4.3.2 Insecticide resistance management of *Tuta absoluta*

The discovery and development of novel insecticides have become more costly and difficult than ever before (Guedes *et al.*, 2019). Therefore, to prolong the efficacy of new and existing insecticides it is crucial that strategies aiming to delay resistance evolution are applied in all agricultural settings (Sparks & Nauen, 2015). Insecticide resistance management (IRM) relies on two components, reducing the selection pressure, and avoiding the selection of resistance mechanisms (Bielza, 2008). Selection pressure on a pest population can be decreased by implementing IPM programmes that make use of different control strategies to effectively manage a pest population (Biondi *et al.*, 2018; Guedes & Picanço, 2012). The application of these alternative non-chemical control strategies could suppress the pest population sufficiently, leading to a reduction in the number of insecticide applications and a decrease in the selection pressure exerted on a pest population (Guedes *et al.*, 2019).

The rotation/alteration of insecticides with different modes of action (MoA) or insecticides affected by different resistance mechanisms is an integral tactic used in IRM to limit the selection of resistance alleles (Guedes & Picanço, 2012; Guedes *et al.*, 2019). However, if the cross-resistance patterns and resistance mechanisms are not identified, the MoA classification scheme developed by IRAC should be followed for effective rotation (Guedes *et al.*, 2019; Sparks & Nauen, 2015). The basic rule when rotating insecticides based on MoA is to avoid the application of the same MoA on consecutive generations of a pest (Guedes *et al.*, 2019). Stakeholders collaborated with IRAC-Spain to develop a MoA rotational scheme (Fig. 1.3) for use in chemical control strategies against *T. absoluta*. In accordance with this rotational scheme, only one MoA can be applied within a treatment window, which, in the case of *T. absoluta* is 30 consecutive days (minimum duration of a single generation of the pest) (IRAC-Spain, 2009). This approach requires a minimum of three effective insecticide MoA groups but ideally more registered MoA groups should be included for optimal delay of resistance evolution (IRAC-Spain, 2009). There are currently nine active ingredients, belonging to five insect resistance action committee (IRAC) groups, registered for the control of *T. absoluta* in South Africa (IRAC, 2017) (Table 1.1).

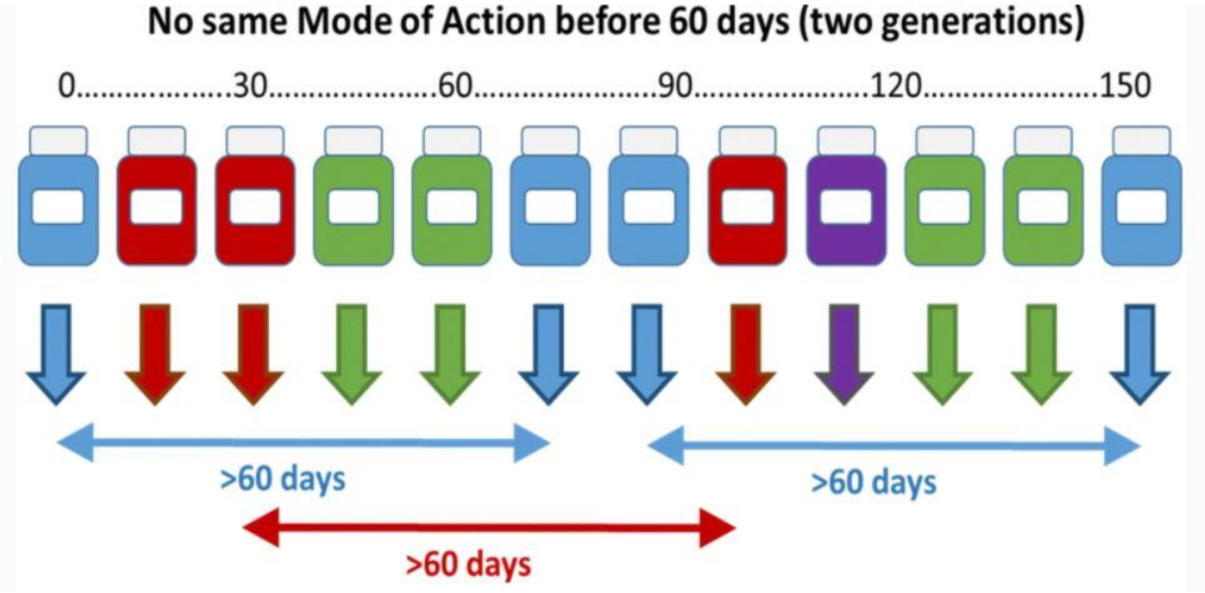

Figure 1.3 Insecticide treatment windows established using the minimum generation duration of *Tuta absoluta* (30 days). Each colour represents a different MoA. Applications of the same MoA are only possible within a treatment window. Different MoA should be selected for application in the second and third treatment windows. The example above is a rotational scheme that consist of four MoA (From Guedes *et al.*, 2019).

1.4.3.3 Systemic insecticides

Systemic insecticides (SI) are substances that, when applied to the plant either as a foliar spray or to the soil/growth medium, are absorbed and translocated to other plant parts via the vascular system and incorporated into plant tissue (Bennett, 1949; Sanchez-Bayo *et al.*, 2013). Target insect species get into contact with systemic insecticides mainly through feeding on treated plants (Sanchez-Bayo *et al.*, 2013). This is in contrast to contact insecticides, which are usually taken up through the insect’s cuticle (Sanchez-Bayo *et al.*, 2013). Systemic insecticides allow for the control of insect species that complete some part or all of their life cycle in plant parts that are largely inaccessible to contact insecticides (Jeppson, 1953). Absorption and translocation are two important factors associated with the efficacy of SI and can be impacted by various plant properties (e.g. species, age, growth stage, growth rate, etc.), environmental conditions (e.g. light intensity, temperature and relative humidity) and soil/growth media properties (e.g. condition, type and components) (Byrne *et al.*, 2010; Cloyd *et al.*, 2011). For the active ingredient of a SI to be successfully absorbed by the plant it must be water-soluble to a certain degree (Hollingworth & Treacy, 2006). Systemic insecticides with a lower water-solubility might persist longer but might not be as effective due to slower mobility (Byrne *et al.*, 2010). On the other hand, highly water-soluble SI may result in the rapid control of insect pests, but might not provide sufficient long-term protection due to a shorter residual activity (Cloyd *et al.*, 2011). The two most important properties that regulate the translocation of SI are acidity (pKa = acid dissociation constant) and lipophilicity (log P_{oct} = octanol-water partition coefficient) (Inoue *et al.*, 1998; Vryzas, 2016). Compounds with intermediate acidity and lipophilicity are particularly effectively translocated (Inoue *et al.*, 1998; Vryzas, 2016).

The application of systemic insecticides not only enables the control of inaccessible pests but also provides protection for an extended period of time, allowing for the multi-generational suppression of pest populations (Byrne *et al.*, 2010; Cloyd *et al.*, 2011; Jeppson, 1953). Reduction in the number of insecticide applications also results in reduced input costs, increased worker safety and less direct negative impacts on beneficial arthropods (Cloyd, 2010; Cloyd *et al.*, 2011). In addition, when applied as a soil application, systemic insecticides are less vulnerable to UV degradation (Cloyd *et al.*, 2011).

Despite the benefits mentioned above, there are several concerns associated with the application of SI. Insecticides exert a selection pressure that favours the survival of resistant genotypes, resulting in reduced efficacy (Biondi *et al.*, 2018; Guedes & Picanço, 2012; Roditakis *et al.*, 2009; Wang & Wu, 2012). The exposure of an insect population to a SI with a long residual period is the equivalent of the repeated application of an insecticide with the same MoA, which could increase the possibility for resistance evolution (Margaritopoulos *et al.*, 2007; Van Leeuwen *et al.*, 2005; Van Leeuwen *et al.*, 2006). Systemic insecticides are translocated to all plant tissues (Lue *et al.*, 1984; Meher *et al.*, 2010), including the pollen and nectar of flowers which could have negative effects on pollinators (Sanchez-Bayo *et al.*, 2013). Natural enemies such as predators and parasitoids could be affected by feeding on pest individuals contaminated with the active ingredient (secondary poisoning) of a

SI (Cloyd & Bethke, 2011; Sanchez-Bayo *et al.*, 2013). In the case of bees and natural enemies, it is not so much the low concentrations of SI that they are exposed to, but rather the length of exposure period that results in population decline (Sanchez-Bayo *et al.*, 2013). The build-up of low residue levels ingested repeatedly over time eventually leads to a delayed toxic effect in populations of these beneficial insects (Tennekes & Sanchez-Bayo, 2012). Additionally, natural enemies of the pest population may also be indirectly impacted by the decrease in prey/host population levels as a result of the long persistence of SI (Cloyd & Bethke, 2011).

1.4.3.4 Systemic activity of spinosad

Spinosad is the mixture of macrocyclic lactones, spinosyn A (major component) and spinosyn D (minor component). Spinosad is also the common name of the active ingredient that is present in several insecticidal products used globally (Cleveland *et al.*, 2001; Sparks *et al.*, 1995; Van Leeuwen *et al.*, 2005). Spinosad is a macrolide isolated from the fermentation of the soil actinomycete, *Saccharopolyspora spinosa* (Mertz & Yao) (Pseudonocardiales: Pseudonocardiaceae) (Thompson *et al.*, 2000). With its unique mode of action, *viz*. depolarizing nicotinic acetylcholine and γ-aminobutyric acid (GABA) receptor neurons (Kirst, 2010; Salgado *et al.*, 1998; Sparks *et al.*, 2012; Thompson *et al.*, 2000), spinosad displays rapid ingestion and contact activity to insect species from the following orders: Coleoptera, Diptera, Hymenoptera, Isoptera, Lepidoptera and Thysanoptera (Bret *et al.*, 1997; Cleveland *et al.*, 2001; Sparks *et al.*, 2012).

Altough only registered to be administered as a foliar spray, Dow AgroSciences reported limited root uptake and translaminar activity of spinosad (Sparks *et al.*, 1999). In 2005, the systemic activity of spinosad was confirmed when administered as a drench to the soil/growth substrate of tomato plants (Van Leeuwen *et al.*, 2005). The drenching of tomato plants grown in rockwool with spinosad resulted in the effective control of *Tetranychus urticae* (Koch) (Acari: Tetranychidae), *Trialeurodes vaporariorum* (Westwood) (Hemiptera: Aleyrodidae) and *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) (Van Leeuwen *et al.*, 2005; Van Leeuwen *et al.*, 2006). Dosages of 1mg a.i./plant resulted in mortality rates exceeding 99% for both *T. urticae* and *T. vaporariorum*, while 5 mg a.i./plant was sufficient to control all third-instar *S. littoralis* larvae (Van Leeuwen *et al.*, 2005; Van Leeuwen *et al.*, 2006). According to Van Leeuwen *et al.* (2005), the approved application rates of spinosad corresponds to between 1 and 10 mg a.i./plant, which means that all three these pests should be effectively controlled for a long period at the maximum application rate of 10 mg a.i./plant. The concentration of spinosad in tomato leaves steadily declines over time, with a significant reduction in control recorded only 25 days after drench application (Van Leeuwen *et al.*, 2005).

The root uptake of spinosad, applied as a soil drench to tomato, was reported to be much higher in a rockwool substrate when compared to other substrates such as black earth, sand, sand-clay- and peat-clay mixtures (Van Leeuwen *et al.*, 2005; Van Leeuwen *et al.*, 2006). This was ascribed to the following properties of rockwool, viz. its high pH (ranging between 7 – 8), lower rate of bacterial breakdown of the active ingredient and lower sorption compared to other fine textured substrates (Thompson *et al.*, 2002; Van Leeuwen *et al.*, 2005).

1.4.4 Biological control

1.4.4.1 Parasitoids

The use of parasitoids as biological control agents against *T. absoluta* have been studied extensively in its native South America (Desneux *et al.*, 2010). Nearly 100 hymenopteran species have been recorded in association with *T. absoluta* (Desneux *et al.*, 2021). However, only three of these species were reported to be associated with *T. absoluta* in both its native range and newly invaded territories, *viz*. *Trichogramma dendrolimi* (Matsumura) *Trichogramma exiguum* (Girault) and *Neochrysocharis formosa* (Westwood) (Ferracini *et al.*, 2019).

Egg parasitoids, the *Trichogramma* (Hymenoptera: Trichogrammatidae) species complex, are preferred for the control of *T. absoluta* due to their ability to rapidly reduce the population size, even before damage is caused to the crop. Pupal and larval parasitoids only control the target pest after some damage has already been caused (Newton, 1998). These trichogrammids are therefore the most promising with records of at least 10 species throughout South America (Lewis *et al.*, 2003).

Effectiveness of these parasitoids to limit *T. absoluta* populations in caged tomato plants was reported by Oliveira *et al.* (2017), with parasitism and emergence rates of 29.6% and 65.9% for *Trichogramma achaeae* and 6.1% and 39.3% for *Trichogramma cordubensis*, respectively. Under greenhouse conditions, parasitism rates and damage reduction exceeding 90% were reported in *T. absoluta* following the release of *T. achaeae* (Cabello *et al.*, 2009; Oliveira *et al.*, 2017).

Mass-rearing programs have been established for several *Trichogramma* species that resulted in these agents being readily available, enabling wide-spread use in inundative biological control (Consoli *et al.*, 2010). To date, only Egypt and Tunisia have used *Trichogramma* parasitoids in the augmentative biological control of *T. absoluta*. In South Africa, only *Trichogrammatoidea cryotophlebiae* is commercially available for the control of *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae) (Newton & Odendaal, 1990), but no information is available on its efficacy against *T. absoluta*.

Only three larval parasitoids of *T. absoluta* have been considered for use in IPM programs, *viz*. *Dolichogenidea gelechiidivoris* (Marsh) (Hymenoptera: Braconidae), *Necremnus tutae* (Ribes and Bernardo) (Hymenoptera: Eulophidae) and *Pseudapanteles dignus* (Muesebeck) (Hymenoptera: Braconidae) (Desneux *et al.*, 2021). However, these parasitoids are currently only incorporated for use in conservation biological control programs (Desneux *et al.*, 2021). The majority of naturally occurring parasitism in commercial fields has been attributed to *D. gelechiidivoris* in Chile (Larrain, 1987) and *P. dignus* in Argentina (Sánchez *et al.*, 2009). Studies by Aigbedion-Atalor *et al.* (2020) on *D. gelechiidivoris* are currently focused on developing a classical biological control program for the control of *T. absoluta* in sub-Saharan Africa.

1.4.4.2 Predators

To date, approximately 60 species of generalist arthropod predators have been reported to prey on *T. absoluta* (Desneux *et al.*, 2021). Of these predatory species, approximately 16% have been recorded outside South America in newly invaded European countries (Ferracini *et al.*, 2019). According to Miranda *et al.* (1998), these naturally occurring predatory species play an integral role in the regulation of *T. absoluta* in its native South America and could be accountable for mortalities of up to 79.4% in larvae and 5% in eggs. Therefore, the conservation of naturally occurring predatory Miridae species may be a key component in the integrated management of *T. absoluta* (Arnó *et al.*, 2018; Jaworski *et al.*, 2015).

In Europe, two endemic zoophytophagous mirid species, *Macrolophus pygmaeus* (Rambur) (Hemiptera: Miridae) and *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae), that were already commercially produced and released mainly for the control of whiteflies (Alomar *et al.*, 2006; Gerling *et al.*, 2001) have been reported to actively prey on eggs and larvae of *T. absoluta* (Mollá *et al.*, 2009; Urbaneja *et al.*, 2009). Starved *M. pygmaeus* and *N. tenuis* were able to prey on over 30 *T. absoluta* eggs daily in laboratory experiments (Urbaneja *et al.*, 2009). Arnó *et al.* (2009) reported predation of more than 100 *T. absoluta* eggs per adult *M. pygmaeus* per day. Although both mirid species actively fed on all *T. absoluta* larval instars they preferentially preyed on smaller first-instar larvae, consuming approximately two larvae per day (Urbaneja *et al.*, 2009). Results from semi-field experiments conducted in greenhouses by Mollá *et al.* (2009) using *M. pygmaeus* against *T. absoluta*, showed a reduction in leaflet and fruit infestation of up to 76% and 56% respectively. *Nesidiocoris tenuis* reduced the number of *T. absoluta* eggs and the percentage area mined by larvae by 83.25% and 89%, respectively (Varshney & Ballal, 2017).

The biological pest control community was initially reluctant to mass release these mirid species due to fears of increased phytophagy and plant injury caused by these omnivorous mirids when animal food is scarce (Albajes & Alomar, 1999; Castañé *et al.*, 2011; Sanchez *et al.*, 2018; Sánchez & Lacasa, 2008). However, both mirid species are now commercially reared and released primarily for the control of whiteflies and *T. absoluta* worldwide (Biondi *et al.*, 2018; Urbaneja *et al.*, 2012).

1.4.4.3 Microbial biological control agents

Several microbial biocontrol agents (MCAs) including bacteria, viruses, and fungi are lethal to *T. absoluta* (Desneux *et al.*, 2021). These MCAs are usually safer for the environment, humans and other vertebrates in comparison to synthetic insecticides, and are more suited for integrated use with arthropod natural enemies (Gonzalez-Cabrera *et al.*, 2011; Mollá *et al.*, 2011).

1.4.4.3.1 Viruses as entomopathogens

Despite the large diversity in insect viruses, only those belonging to the genera *Alphabaculovirus* and *Betabaculovirus* of the family Baculoviridae have been developed and registered as microbial insecticides (Erlandson, 2008). Baculoviruses are a large group of rod-shaped double stranded dsDNA viruses with circular genomes (Herniou & Jehle, 2008). These are used in over 50 registered products for the control of a variety of pests worldwide (Moscardi *et al.*, 2011). To date in South Africa, baculovirus isolates are registered only for the control of *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae) and *H. armigera* (Knox *et al.*, 2014).

Despite the high potential of baculoviruses in controlling other lepidopteran species, its use in the control of *T. absoluta* larvae has been poorly studied (Gómez Valderrama *et al.*, 2018). Tiba *et al.* (2018) reported the pathogenicity of *Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae) granulovirus (PhopGV) isolates to *T. absoluta*, but were not able to elucidate on their effectiveness in controlling larvae of this pest. Gómez Valderrama *et al.* (2018) on the other hand, reported high efficacy of two PhopGV isolates against *T. absoluta* larvae in laboratory experiments with a significant potential to be used as active ingredients for new viral biopesticides against the tomato leaf miner.

1.4.4.3.2 Bacteria as entomopathogens

The gram-positive, aerobic bacterium, *Bacillus thuringiensis,* is one of the most important microorganisms with entomopathogenic activity against certain insect orders (Alsaedi *et al.*, 2017). Different subspecies of this bacterium are widely used to control lepidopteran pests in vegetables, including *T. asboluta* (Desneux *et al.*, 2021). During the sporulation phase, *B. thuringiensis* produces a specific crystalline protein inclusion, called Cry-endotoxins (δ endotoxin) (Alsaedi *et al.*, 2017). These crystals contain a protein sequence that is highly toxic to numerous insect species (Douville *et al.*, 2005; Schnepf *et al.*, 1998), and are often comparable with chemical insecticides with regards to their efficacy (Desneux *et al.*, 2021). Gonzalez-Cabrera *et al.* (2011) found that Bt-based insecticides were highly effective in controlling *T. absoluta* larvae. When plants infested with first-instar *T. absoluta* larvae were sprayed weekly with a Bt-based insecticide, leaflet damage was approximately 90-fold lower than that of untreated control plants, with reduced susceptibility observed in later larval instars (Gonzalez-Cabrera *et al.*, 2011). Similarly, Medeiros *et al.* (2006) reported effective control of *T. absoluta* with Bt-products during Spanish outbreaks of the pest.

During its vegetative stage*, B. thuringiensis* produces and secretes the vegetative insecticidal protein (Vip) (Sellami *et al.*, 2015). The Bt Vip3Aa16 protein was reported to have a higher potency than the δ-endotoxins of Bt, when the toxicity of both were evaluated against *T. absoluta* (Sellami *et al.*, 2015).

1.4.4.3.3 Fungi as entomopathogens

Unlike viruses and bacteria, EPFs infect insects by direct cuticle penetration (Bilgo *et al.*, 2018). An EPF infection commences with the adhesion of conidia to the cuticle of a susceptible host, which is a passive process aided by wind or water (Sinha *et al.*, 2016). After adhesion, conidia germinate, with further growth profoundly influenced by the availability of carbon and nutrients at sufficient oxygen, humidity and temperature levels (Litwin *et al.*, 2020; Sinha *et al.*, 2016). Emerged appressoria penetrate the insect’s cuticle through mechanical pressure and enzymatic degradation (Lacey *et al.*, 2015). Subsequently, fungal hyphae start to grow along with the production of secondary metabolites, which result in host paralyses and disruption of physiological processes such as immune responses (Donzelli & Krasnoff, 2016). Death of the insect host is the result of a combination of factors, including the effect of produced toxins, nutrient depletion and mechanical injury to internal organs (Fan *et al.*, 2017; Mascarin & Jaronski, 2016).

Over 100 fungal genera and 750 species have been reported to parasitize living insects (Roberts & Yendol, 1971; Sujeetha & Sahayaraj, 2014) and are categorized in six classes: Basidiomycota, Chytridiomycota, Entomophtoromycota, Oomycetes and the most common Ascomycota (Litwin *et al.*, 2020). However, most of the EPF containing biopesticides used worldwide, contain species belonging to the genera *Beauveria*, *Hirsutellam*, *Isaria*, *Lecanicillium* and *Metarhizium* (Castro *et al.*, 2016; Khan *et al.*, 2012) and are able to infect insects of almost all orders. The most commonly infected host orders are Coleoptera, Diptera, Hemiptera, Hymenoptera, Orthoptera and Lepidoptera (Ramanujam *et al.*, 2014). Their wide host ranges, worldwide distribution and vast ecological niches has resulted in approximately 68% of commercially available EPF products to be based on *B. bassiana* and *M. anisopliae* (de Faria & Wraight, 2007; McGuire & Northfield, 2020; Sujeetha & Sahayaraj, 2014).

Promising results on the efficacy of *B. bassiana* and *M. anisopliae* for the control of *T. absoluta* have been reported. İnanlı *et al.* (2012) reported egg and larval mortalities exceeding 90% when treated with *M. anisopliae*. *Beauveria bassiana* had pathogenicity to *T. absoluta* eggs resulting in mortalities of 67% for eggs (İnanlı *et al.*, 2012). However, *T. absoluta* eggs exposed to *B. bassiana* and M*. anisopliae* at a concentration of 1×10^7 conidia mL^{-1} resulted in a mortality rate of 100% for both fungal species (Abdel-Raheem *et al.*, 2015). Several studies reported *T. absoluta* larvae to be highly susceptible to *B. bassiana* and *M. anisopliae*, with mortalities exceeding 90% (Hammad *et al.*, 2021; Rodríguez *et al.*, 2006; Tadele & Emana, 2017). Contreras *et al.* (2014) provided evidence of the suppressive potential of *M. anisopliae* when conidia were applied as a soil drench to disrupt the life cycle of *T. absoluta* by controlling the pupal phase of the pest.

1.5 Aims and objectives

1.5.1 Study aim

The aim of this study was to investigate the use of biopesticides using novel strategies for the control of *T. absoluta* and the effect of *Wolbachia* on pheromone-based control strategies.

1.5.2 Study objectives

- to investigate the susceptibility *T. absoluta* pupae to EPFs, when applied as a soil drench and their potential effect on female fertility and fecundity.

- to determine whether South African *T. absoluta* populations can reproduce parthenogenetically, and if so, whether it is the result of manipulation by *Wolbachia*.

- to evaluate the *T. absoluta* larval instar-dependant tolerance to spinosad, the efficacy and residual persistence of spinosad drench application for control of *T. absoluta* and the residual persistence of systemic toxicity of spinosad in leaves and fruit.

1.6 Structure of thesis

Chapter 1 provides the general background, aims and objectives, and thesis outline. The literature study provides an overview on the impact of *T. absoluta* on tomato production and the available strategies applied to control this pest. It also highlights the influence of pest behaviour and biology on the effectiveness of these strategies.

Chapter 2 (first article) investigates the susceptibility of *T. absoluta* pupae to EPFs applied as a soil drench and the effect of infection on female fertility and fecundity.

Chapter 3 (second article) investigates the prevalence of *Wolbachia* in South African *T. absoluta*, the genetic diversity and whether or not these populations have the ability to reproduce through parthenogenesis.

Chapter 4 (third article) evaluates *T*. *absoluta* larval instar-dependant tolerance to spinosad, the efficacy and residual persistence of spinosad applied as drench for the control of *T. absoluta* and the residual persistence of systemic toxicity of spinosad in leaves and fruit.

Chapter 5 provides an in-depth discussion of the key findings of the thesis and how these findings contribute to IPM of *T. absoluta*.

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Chapter 2

Susceptibility of *Tuta absoluta* (Lepidoptera: Gelechiidae) pupae to soil applied entomopathogenic fungal biopesticides

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Susceptibility of *Tuta absoluta* (Lepidoptera: Gelechiidae) Pupae to Soil Applied Entomopathogenic Fungal Biopesticides

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Simple Summary: The invasive tomato pinworm is one of the most destructive insect pests of tomato in Africa. The majority of farmers respond to infestations by applying chemical insecticides. However, the overreliance on this control method has deemed several insecticides ineffective due to resistance evolution. It is therefore crucial that integrated approaches are put in place, of which biopesticides play an important role, to mitigate this problem. Amongst the biopesticides, entomopathogenic fungi (EPF) are promising options. EPF applications aimed to control this pest have been highly effective, although the majority are applied against the larval and egg stages. This study investigated the susceptibility of pupae of the tomato pinworm against EPF products (*Beauveria bassiana* and *Metarhizium anisopliae*) when applied as a soil drench. High pupal mortality rates were recorded for all EPF products tested in bioassays and growth tunnel experiments. A significant reduction in fecundity was observed in moths that survived the pupal EPF applications, with no effects on moth fertility. Overall, our findings provide evidence of the suppressive potential when administering EPF conidia as a soil drench to disrupt the life cycle of *Tuta absoluta* for use in integrated pest management programs.

Abstract: Management of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) in greenhouses and under open-field tomato cultivation relies on an integrated approach, largely targeting the egg and larval stages of the pest. However, little to no research has been done on the efficacy of EPFs for control of the pupal stage. The aims of this study were to determine the susceptibility of *T. absoluta* pupae to *Beauveria bassiana* and *Metarhizium anisopliae* spores applied as soil drench treatments, and the possible effects of these treatments on fecundity and fertility of moths. The lethal concentrations (LC_{50} and LC_{80}) of the respective products were estimated in dose-response bioassays by exposing pupae in a soil substrate to different concentrations of EPF products. Emerging moths were paired in different combinations, according to EPF exposure treatments after which fecundity and fertility of females were recorded. Pupae in the soil were effectively controlled by all EPF products in both bioassays as well as in a growth tunnel experiment. The LC_{50} value of the *B. bassiana* oil formulation was significantly lower than that of other treatments. The fecundity of females that were subjected to the *B. bassiana* oil formulation was significantly lower than that of the control treatment. This study showed the potential of soil drench applications of both *B. bassiana* and *M. anisopliae* for control of *T. absoluta* pupae.

Keywords: biopesticide; pest management; pupae; soil application; tomato pinworm

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1. Introduction

Tuta absoluta (Meyrick) (Lepidoptera: Gelechiidae) extended its geographical distribution around the world since it was first reported outside its native range in South America, in 2006. *Tuta absoluta* was reported for the first time in the eastern parts of Spain from where it rapidly spread through Europe, the Middle East, Asia, and Africa [1–8].

The application of synthetic chemical insecticides is the most commonly used practise to control *T. absoluta* infestations in tomato crops. However, the overreliance on insecticides exerts selection pressure that favours the survival of resistant genotypes, evidently leading to the evolution of resistance and reduced efficacy of insecticides [9–12]. It is therefore crucial that an integrated approach that includes biological control, pheromone-based control methods, and biopesticides, in combination with synthetic insecticides when needed, is developed for the management of *T. absoluta* [9,13–17].

Biological control through the release of parasitoids and predators, as well as application of biopesticides have been investigated as management options for *T. absoluta* [18]. Amongst these, entomopathogenic fungi (EPFs) represent one of the most promising options. EPFs have been reported to be highly efficacious against a wide range of pests, especially subterranean pests [19,20]. Both *Beauveria bassiana* (Hypocreales: Cordycipitaceae) and *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae) have been reported to be effective against *T. absoluta* [21–23]. However, these studies largely focused on the efficacy of these mycoinsecticides against the egg and larval stages of this pest. Only one study evaluated the efficacy of fungal pathogens against *T. absoluta* pupae in the soil and reported effective control with an *M. anisopliae* formulation applied as a soil drench [24].

The aims of this study were to determine the susceptibility of *T. absoluta* pupae to *B. bassiana* and *M. anisopliae* in powder and oil formulations applied as soil drench treatments, and the possible effects of these EPFs on fecundity and fertility of moths that emerge from surviving EPF-treated pupae.

2. Materials and Methods

2.1. Insect Rearing

A rearing colony was established from a *T. absoluta* population collected from infested tomato fields at Mareetsane in the North West Province of South Africa (S 26°41′52″; E 25°26′24″). In the laboratory, the infested leaves were placed onto a plastic mesh (hole diameter = 2 cm) which was suspended from the roof of an insect rearing cage (100 cm × 100 cm × 150 cm), above healthy potted tomato plants (cv. Monica). This allowed larvae to migrate from the sampled leaves onto the potted tomato plants to complete their life cycle. Adults were collected on a daily basis from this rearing cage and transferred to oviposition cages that contained healthy potted tomato plants. Cotton swabs soaked in a 10% sugar solution were provided in Petri dishes at the bottom of the cages, as an energy source for moths. Plants were replaced every second day to ensure that the eggs recovered from these plants were of a similar age and that they would hatch at approximately the same time. Mass rearing of this *T. absoluta* population was done for several generations as described above.

To use pupae of a similar age in bioassays, *T. absoluta* infested leaves were removed from plants and dissected to recover larvae, 14 days after oviposition. Third- to fourth-instar larvae were removed and placed in plastic, ventilated containers (15 cm × 10 cm × 7 cm) and provided with tomato shoots with leaves, to allow for larvae to complete their development until pupation. Rearing and oviposition cages were maintained at 26 ± 1 °C, 65% RH, and at a 14 L: 10D photoperiod.

2.2. Entomopathogenic Fungi

Two commercial products, a wettable powder (WP) and an emulsifiable suspension (ES), of *M. anisopliae* (MET-WP and MET-ES) and *B. bassiana* (BB-WP and BB-ES), were used in the bioassays. Only the BB-WP product is currently registered for control of *T. absoluta* larvae on tomato in South Africa. The commercial EPF products with all complementary information are listed in Table 1.

Table 1. Commercial entomopathogenic fungi used and their respective target pests as per product label.

Commercial Name and Manufacturer	ACTIVE Ingredient	Experimental Name	Batch Concentration	Target Pest Registration
Metarril WP E9 Koppert SA (Pty) (Falcon Ln, Lanseria, 1739, South Africa)	*Metarhizium anisopliae* (Metsch.) Sorok., strain E9	MET-WP	2.00 × 10^9 conidia/g	*Mahanarva fimbriolata* (Stål) (Hemiptera: Cercopidae)
Real Metarhizium 69 Real IPM (SA) Ltd. (Grabouw, 7160, South Africa)	*Metarhizium anisopliae* (Metsch.) Sorok., strain ICIPE 69	MET-ES	3.23 × 10^9 conidia/mL	Fruit flies (Diptera) Mealybug (Hemiptera) Weevils (Coleoptera) Thrips (Thysanoptera) Whiteflies (Hemiptera)
Eco-Bb® Plant Health Products (Pty) (Nottingham Road, 3280, South Africa)	*Beauveria bassiana* (Bals.) Vuill., strain R444	BB-WP	2.20 × 10^9 conidia/g	*Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) *Tetranychus* spp. (Acari: Tetranychidae) *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae) *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) Whiteflies (Hemiptera)
Real Beauveria Real IPM (SA) Ltd. (Grabouw, 7160, South Africa)	*Beauveria bassiana* (Bals.) Vuill., strain BB02	BB-ES	2.74 × 10^7 conidia/mL	Thrips (Thysanoptera)

2.3. *Laboratory Dose-Response Bioassays*

Laboratory bioassays were conducted to expose *T. absoluta* pupae to *B. bassiana* and *M. anisopliae* conidia, at different concentrations. The bioassays were conducted in 275 mL plastic containers (height: 9 cm, diameter: 7 cm) fitted with mesh (diameter: 3 cm) on the lids. Three holes (diameter: 4 mm) at the base of each container allowed for drainage of excess water. A commercial substrate (Seedling mix by Culterra, Johannesburg, South Africa) was autoclaved and used to fill the plastic containers to a depth of 6 cm. Distilled water (30 mL) was added as a drench to wet the substrate and initiate drainage. Pupae for the bioassays were collected daily from the rearing containers. Ten 2 to 3-day old pupae were placed onto the substrate in each container and covered with a thin layer (±3 mm) of the substrate to simulate natural pupation conditions.

Serial dilutions for each of the *M. anisopliae* and *B. bassiana* biopesticides were prepared in 1 L water, of which 40 mL was added as a drench to the respective containers. The treatments of each bioassay consisted of nine EPF concentrations per product (MET-WP: 5×10^8–1.2×10^{10}; MET-ES: 1.615×10^7–2.0672×10^9; BB-WP: 2.75×10^8–2.2×10^{10}; BB-ES: 5.48×10^5–7.124×10^7 viable conidia per litre) and a control. Distilled water was applied as the control for wettable powder products whereas, for emulsifiable suspensions, the control consisted of the products without EPF conidia (oil mixture). Each treatment was replicated four times and each container with pupae served as a replicate. Containers were maintained at 26 ± 1 °C, 65% RH with a 14 L: 10D photoperiod. The number of moths that emerged per container was recorded at three-day intervals for a period of three weeks. The mortality of pupae was calculated and expressed as a percentage. Mortality data were used to estimate the lethal concentrations for each EPF formulation.

2.4. *Growth Tunnel Experiment*

The ability of four EPF biopesticides to control *T. absoluta* pupae, when administered as a soil drench was evaluated in a greenhouse tunnel. The plant growth tunnel was covered with light defused plastic with 75% light transmission at the top and 40% white shade netting on the sides up to 1.25 m from ground level. Relative humidity and temperature were not regulated. IButton® (ColdChain ThermoDynamics, Fairbridge technologies, Johannesburg, South Africa) wireless data loggers which were placed at various points throughout the plant tunnel recorded relative humidity and temperature at 30 min intervals throughout the experiment. The mean minimum and maximum percentage relative humidity (RH) and temperature during the experiment were measured as 31.45%; 16.38 °C and 82.25%; 36.61 °C with an overall mean RH of 59.80% and temperature of 24.56 °C.

Tomato seedlings were planted in 2 L pots (diameter: 16 cm) filled with a 4:1 soil:compost mixture, up to 2 cm from the rim. The soil consisted of 1.3% clay, 78% sand, 1.2% silt, with a pH of 6.49. The compost used was commercially available (Culterra, Johannesburg, South Africa). Ten 2- to 3-day-old pupae were placed onto the soil mixture in each pot and covered with a thin layer (±3 mm) of the substrate to simulate natural pupation conditions.

The four EPF products were applied as soil drench treatments at rates estimated as the number of conidia/cm^2 which resulted in 80% mortality of the population (LC_{80}) as determined in the bioassay described above. Distilled water was applied as the control treatment for wettable powder products whereas for emulsifiable suspensions the control consisted of the products without EPF conidia (oil mixture). The EPF treatments were applied at a volume of 200 mL/pot. The pots were covered with insect-proof bags made of organza material (height: 70 cm, and width: 35 cm), fitted with an elastic band at the bottom to prevent the escape of moths and to enable the recording of the numbers of eclosed moths at three-day intervals over a twelve-day period. The mortality of pupae was calculated and expressed as a percentage. Each treatment was replicated 10 times and each pot with 10 pupae served as a replicate. The experimental design was a randomised complete block design.

2.5. *Effect on Fecundity and Fertility*

The fecundity and fertility of moths of the pupae that survived the EPF treatments were determined in a laboratory bioassay, similar to that described above. However, in this bioassay, only the two EPF products (MET-ES and BB-ES) that were more effective in controlling *T. absoluta* pupae in both the laboratory bioassay and the tunnel experiment, were applied as drench treatments at the concentrations estimated as the LC_{80} per product. Six-day-old pupae were used instead of 2 to 3-day-old pupae to ensure that a sufficient number of moths emerged to successfully determine the effect of EPF biopesticides on fecundity and fertility of moths. Pupae were sexed by examining the position of genital openings [25]. Male and female pupae were exposed to EPF formulations separately.

The number of moths that emerged was recorded daily over a 7-day period. Male and female moths that emerged on the same day were paired and single pairs were placed into ventilated oviposition cages (height: 15 cm, diameter: 9 cm). A fresh tomato shoot, inserted into a plastic tube (height: 5.5 cm, diameter: 1.2 cm) filled with water, was provided as an oviposition substrate.

The pairing of moths was done according to antecedent exposure of the pupae to the respective treatments, and also included crosses between moths that emerged from treated and untreated pupae (Table 2). Fecundity and fertility of moths from the different treatment combinations were recorded daily at which time a fresh tomato shoot was provided until the females died.

Table 2. Treatment combinations of moths that emerged from pupae exposed to emulsifiable suspensions of *Metarhizium anisopliae* and *Beauveria bassiana*.

***M. Anisopliae*-ES**	***B. Bassiana*-ES**
M. anisopliae (Male) × *M. anisopliae* (Female) MM × MF	*B. bassiana* (Male) × *B. bassiana* (Female) BM × BF
M. anisopliae (Male) × Control (Female) MM × CF	*B. bassiana* (Male) × Control (Female) BM × CF
Control (Male) × *M. anisopliae* (Female) CM × MF	Control (Male) × *B. bassiana* (Female) CM × BF
Control	
Control (Male) × Control (Female) CM × CF	

Dead moths were removed from the oviposition cages to determine their infection status. These moth cadavers were individually surface-sterilized by dipping them first in 0.5% NaOCl (active chlorine) which contained 0.05% Tween 80 for 1 min, followed by 2 min in 70% ethanol, followed by two washes of 1 min each with sterile water [22]. Sterilized cadavers were incubated in Petri dishes (6 cm diameter) lined with moist filter paper and maintained at 26 ± 1 °C in total darkness for two weeks to allow for fungal growth (mycosis test). This allowed us not only to determine the incidence of moths with mycoses but also to determine whether EPFs were transferred horizontally to unexposed control moths. For treatments with *B. bassiana* conidia, external characteristically white to cream-coloured powdery sporulation on the insect integument [26] is indicative of infection, while a characteristically green-coloured sporulation indicated *M. anisopliae* infection [27].

2.6. Data Analysis

Abbott's formula was used to correct the data for pupal mortality in the control treatment [28] in the laboratory bioassays and the growth tunnel experiment. Corrected mortality data from the respective laboratory dose-response bioassays were subjected to probit analysis and the relative potency ratio among responses was calculated using PoloSuite® software (LeOra Software LLC, version 1.8; Northampton Northamptonshire NN1 2JL, UK). Responses were considered to be significantly different when the 95% confidence interval of the relative potency ratio did not include the value 1.

Fecundity, fertility, percentage moth emergence and corrected percentage mortality (growth tunnel) data were tested for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test). Fecundity, percentage moth emergence, and log-transformed fertility data met these assumptions and were subsequently subjected to analyses of variance, using TIBCO Statistica™ 13.3 (TIBCO Software Inc. 2017, Palo Alto, CA, USA) [29]. Treatment means were separated using the Unequal N HSD-test at $p = 0.05$. Mycosis followed a binomial distribution (infected or not infected), and data were therefore analysed by means of binomial distribution tests. Bonferroni correction was used to adjust for multi-mean comparisons.

Corrected percentage mortality data obtained from the growth tunnel experiment were neither normally distributed nor homoscedastic, therefore the data were analysed by performing the Kruskal-Wallis test using Statistica™ 13.3 [29].

3. Results

The responses of *T. absoluta* pupae to the respective EPF formulations fitted the log (dose)/probit (mortality) model at $p < 0.05$ (Table 3). The slope coefficients of the four formulations ranged between 1.1 and 1.7, suggesting a homogenous response to the different mycoinsecticide.

The estimated LC_{50} values of the respective treatments differed significantly according to the 95% confidence intervals of the relative potency ratio ($p < 0.05$) (Table 3). The oil formulations of both EPF species provided 100% control of pupae (Figure 1), and the LC_{50} values of the oil-based formulations were also significantly lower compared to the powder formulations of both products (Table 3). The BB-ES product was the most effective with an estimated LC_{50} value of 1.87×10^7 viable conidia L^{-1}. When the BB-WP product was applied as a drench according to the recommended field rate for foliar application against *T. absoluta* (2.2×10^9 viable conidia L^{-1}) to control larvae, the estimated mortality of pupae was approximately 60% (Figure 1).

Table 3. Log-dose probit mortality data for *Tuta absoluta* pupae treated with different entomopathogenic fungal products and corrected percenta

Treatment	n [a] (df)	LC_{50}	FL (95%) [b]	LC_{80}	FL (95%)	Slope	SE
BB-ES	280 (5)	1.87×10^{7}	1.3×10^{7}–2.90×10^{7} a	1.09×10^{8}	6.25×10^{7}–2.50×10^{8}	1.10	0.13
MET-ES	240 (4)	1.38×10^{8}	9.40×10^{7}–1.88×10^{8} b	4.31×10^{8}	3.15×10^{8}–6.38×10^{8}	1.70	0.19
MET-WP	329 (6)	3.84×10^{9}	2.82×10^{9}–5.5×10^{9} d	1.73×10^{10}	1.05×10^{10}–4.25×10^{10}	1.29	0.18
BB-WP	350 (5)	1.49×10^{9}	1.02×10^{9}–2.0×10^{9} c	5.26×10^{9}	3.9×10^{9}–7.34×10^{9}	1.53	0.16

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[a] n = number of pupae tested; FL = fiducial limits; LCs in conidia L^{-1}. [b] FLs within the same column followed by the same letter are not significantly differen
dose–mortality response. [d] Number of pupae tested in growth tunnel experiment. Mean % corrected mortality caused by the respective treatments are not signifi
in the column (Kruskal-Wallis followed by Dunn's multiple comparison test; $p < 0.05$).

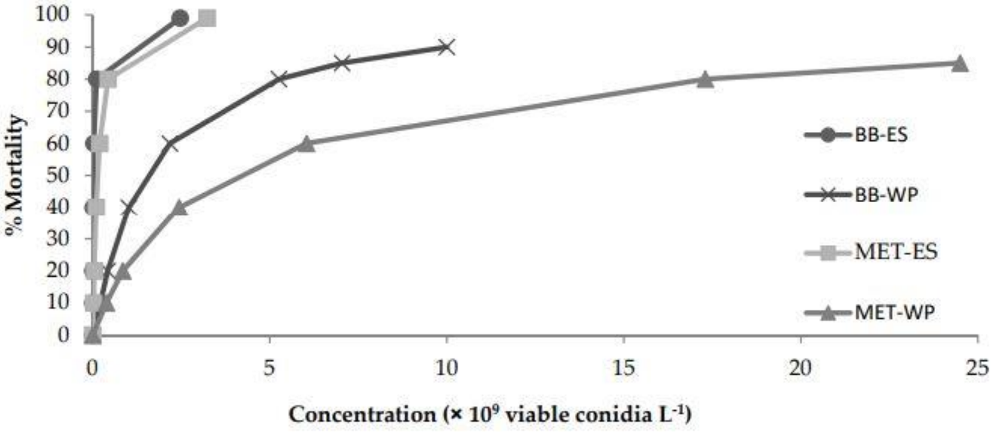

Figure 1. Estimated percentage mortality of *Tuta absoluta* pupae subjected to different concentrations of four entomopathogenic fungal formulations.

High pupal mortality (68–98.8%) occurred where EPF products were applied at their respective LC_{80} concentrations under growth tunnel conditions. Pupal exposure to BB-ES resulted in significantly higher mortality (98.8%) compared to mortality of those treated with BB-WP (68.8%) (H(3) = 13.67; $p < 0.05$) (Table 3). Pupal mortality caused by *B. bassiana* (oil-based and wettable powder) and *M. anisopliae* (oil-based and wettable powder) did not differ significantly (Table 3).

Fecundity of females was significantly affected by antecedent exposure to EPFs (F = 3.02; df = 6, 61; $p < 0.05$). When males from EPF exposed pupae mated with females from *M. anisopliae* treated pupae, significantly fewer eggs were laid, with 33.0 and 35.6 eggs per female for the *B. bassiana* and *M. anisopliae* treatment respectively, compared to the fecundity of females of the control treatment (85.6 eggs per female) (Table 4). In addition, a significant reduction in fecundity was recorded in females exposed to *B. bassiana*, regardless of the EPF exposure status of the male (33.9 eggs per female) (Table 4). Mating of male moths, exposed during the pupal stage to either *B. bassiana* or *M. anisopliae*, with females from unexposed pupae did, however, not affect fecundity of females (57.7 and 57.9 eggs per female, for the respective treatments) (Table 4). Fertility was not affected by the EPF exposure status of the moths since there was no significant difference in fertility of the respective treatment pairs (F = 0.65; df = 6, 49; $p = 0.69$) (Table 4). High fertility (between 92 and 97%) was recorded for all pairs, regardless of their antecedent exposure to *B. bassiana* or *M. anisopliae* or no exposure to EPFs (Table 4).

Significantly fewer moths emerged from six-day-old pupae treated with BB-ES, compared to MET-ES treated pupae as well as from untreated control pupae, with no difference in emergence between the latter two treatments (F = 28.76; df = 2, 44; $p < 0.001$) (Table 5). Mycosis of moths from the BB-ES treatment was also significantly higher (82%) compared to the MET-ES and control treatments (F = 145.27; df = 2, 139; $p < 0.001$) (Table 5). The cadavers of moths that emerged from the uninfected pupae did not show any signs of mycosis, even after these moths mated with moths that emerged from pupae that were treated with either *B. bassiana* or *M. anisopliae* (Table 5).

Table 4. Mean fecundity and fertility (±SE) of EPF-treated and untreated *Tuta absoluta* females after mating with either EPF-treated or untreated males.

Treatment	n	Mean Fecundity per Female	Mean Percentage Fertility
BM × BF	10	33.00 ± 9.59 b *	93.99 ± 2.48 a *
BM × CF	9	57.67 ± 17.40 ab	92.19 ± 2.33 a
CM × BF	10	33.90 ± 9.17 b	92.40 ± 4.43 a
MM × MF	10	35.60 ± 8.06 b	96.08 ± 1.50 a
MM × CF	10	57.90 ± 10.11 ab	95.55 ± 1.63 a
CM × MF	9	67.33 ± 12.03 ab	97.26 ± 1.18 a
CM × CF	10	85.60 ± 11.41 a	96.56 ± 1.22 a

* Means within the same column followed by the same letter are not significantly different $p < 0.05$ (Unequal N HSD). n = Number of pairs. BM = Males treated with BB-ES formulation, BF = Females treated with BB-ES formulation, MM = Males treated with MET-ES formulation, MF = Females treated with MET-ES formulation, CM = Untreated male, CF = Untreated female.

Table 5. Mean percentage moth emergence and mycosis after six-day-old *Tuta absoluta* pupae were treated with EPF products.

Treatments	n [a]	Mean Percentage Moth Emergence [c]	n [b]	Mean Percentage Mycosed Moths [d]
BB-ES	157	31.60 ± 4.82 a	39	82.05 ± 6.22 a
MET-ES	189	65.82 ± 4.32 b	43	6.98 ± 3.93 b
Control	102	80.50 ± 3.20 b	60	0.00 ± 0.00 b

[a] n = Number of pupae tested. [b] n = Number of moths used in mycosis test. [c] Means within the same column followed by the same letter are not significantly different at $p < 0.05$ (Unequal HSD). [d] Means within the same column followed by the same letter are not significantly different at $p < 0.05$ (Binomial distribution tests, followed by Bonferroni correction).

4. Discussion

The application of synthetic insecticides forms the basis of *T. absoluta* management in Africa, although other approaches are also used [30]. It is, however, important that all life stages be targeted to control this pest. Results from this study provide evidence that EPFs can effectively kill a high percentage of *T. absoluta* pupae when applied as a soil drench. This was especially so in the case of the BB-ES formulation, which not only had the lowest estimated LC_{50} value against two-day-old pupae but proved to be fast-acting as it was also able to cause pupal mortality of approximately 70% of six-day-old pupae when they were exposed. The EPF treatments were also highly effective in the growth tunnel experiment, with 98.8% mortality of 2 to 3-day old pupae recorded. The reduced percentage of moths that emerged from pupae in the soil after BB-ES inoculation laid significantly fewer eggs. It, therefore, indicates that BB-ES inoculation to soil containing *T. absoluta* pupae may reduce the population size of subsequent generations of the pest.

For the initial laboratory bioassays, it was necessary to remove all other biotic factors in the soil to effectively evaluate the direct effects of EPF products on *T. absoluta* pupal mortality, therefore a commercially available substrate was autoclaved and used. However, in the growth tunnel experiments, tomato plants were grown in a non-sterile soil-compost mixture. Under these semi-field conditions in the growth tunnel, all EPF products exceeded the expected mortality rate of 80% when administered at their LC_{80}'s as determined in laboratory bioassays, except for the BB-WP product. The higher mortality rates observed in the greenhouse tunnel experiment could therefore be ascribed to the differences in abiotic and biotic factors present in the soil such as soil texture, moisture level, temperature, and the presence of viable soil microbiota [31,32].

Females that were exposed as pupae to *B. bassiana* laid significantly fewer eggs regardless of whether or not males were treated with EPFs, indicating that *B. bassiana* infection in females significantly lowered fecundity. A reduction in fecundity was also observed for *M. anisopliae*, however only when both males and females were infected.

There was, however, no horizontal transmission of fungi between moths when fungi were acquired during the pupal stage, and mating occurred after moth eclosion from these pupae. This absence of direct horizontal transmission from infected to uninfected adults could possibly be ascribed to the adhesion of conidia to the surfaces of pupae and not to the body surfaces of moths emerging from these pupae. The importance of the availability of body surfaces for adhesion of conidia was demonstrated in *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae) females [33]. The latter study reported the ability of male *T. leucotreta* moths to acquire higher numbers of dry *M. anisopliae* conidia than females, to a batch of scales on the hind tibia of males, which is absent in females.

Toxicological data for fungal-based biological insecticides against pupae of *T. absoluta* was also provided by Contreras et al. [24]. The dose-responses recorded in the present study were similar to those obtained by Contreras et al. [24] who evaluated a liquid formulation of *M. anisopliae* conidia against *T. absoluta* pupae in 900 mL containers (15 × 12 × 5 cm). The estimated LC_{50} value converted to conidia/cm^2 for MET-ES (3.59×10^6 conidia/cm^2) was, however, higher than those reported by Contreras et al. [24] for an *M. anisopliae* formulation (ranging between 1.17 and 3.0×10^6 conidia/cm^2) evaluated against different populations of *T. absoluta* in Spain. While the LC_{50} value of *B. bassiana* emulsifiable suspension (4.86×10^5 conidia/cm^2) evaluated in the current study was considerably lower than that reported by Contreras et al. [24].

BB-WP is currently the only fungus-based product registered for control of *T. absoluta* in South Africa, and it is applied as a foliar spray, specifically targeting the larval phase. This study confirmed the efficacy of the BB-WP product with *B. bassiana* strain R444 as an active ingredient against *T. absoluta.* However, to achieve *T. absoluta* pupal mortality higher than 80% with a soil application of the BB-WP product, a high dosage of at least 3.87×10^7 conidia/cm^2 will be required, which is significantly higher when compared to the other products tested.

The pupae treated in the fecundity and fertility bioassays were six days old at the time of treatment to ensure that a sufficient number of moths emerge to efficiently determine the possible effects on the abovementioned parameters. However, it also provided additional data on moth emergence when products were administered to older pupae. Bajracharya and Bhat [34] recorded an average developmental period of 7.11 days for *T. absoluta* pupae at 27 ± 2 °C. The time period available for EPFs to germinate and penetrate through the cuticles of six-day-old pupae was very short (one day). Therefore, differences in virulence between the BB-ES and MM-ES recorded in the case of older pupae could possibly be ascribed to a quicker conidial germination rate and cuticle penetration capacity of the *B. bassiana* (strain BB02) [35]. The percentage of moth emergence recorded when six-day-old pupae were treated with BB-ES and MET-ES compared to 2 to 3-day old pupae were higher as a result of a shorter period of time available to EPFs to cause infection. However, it could still result in a considerable reduction in population size even if EPF products are applied as a soil drench in a field or glasshouse of which *T. absoluta* pupae are of different ages. If BB-ES and MET-ES are applied at LC_{80} rates when most pupae are still three to four days of age, it could result in 98.8% and 90% pupal mortality with a reduction in fecundity of surviving moths of 52.6% and 50% respectively.

Previous studies on the effects of *B. bassiana* and *M. anisopliae* on *T. absoluta* larvae and eggs reported promising efficacy. Laboratory bioassays performed by Abdel-Raheem et al. [36] reported 100% mortality at 1×10^7 conidia mL^{-1} when *T. absoluta* eggs were exposed to *B. bassiana* and *M. anisopliae.* İnanlı et al. [37], who dipped leaves with *T. absoluta* eggs into EPF suspensions, reported that egg mortality exceeded 90% when treated with an *M. anisopliae* product, but mortality of only 67% was recorded when eggs were treated with a *B. bassiana* product. The larval phase was also shown to be highly susceptible to isolates of both *B. bassiana* and *M. anisopliae*, resulting in mortalities exceeding 90% [22,23,38].

Soil serves as the natural ecosystem for EPFs, providing fungi with optimal moisture and temperature conditions and protection against UV radiation [31]. More importantly, soil also serves as the habitat where EPFs come into contact with the soil-dwelling life stages of insects. Consequently, the persistence of entomopathogenic fungi in the soil is a requirement for successful control. Garrido-Jurado et al. [39] reported that the availability of *B. bassiana* and *M. anisopliae* is significantly affected by soil properties, although no significant effects were recorded on the pathogenicity of EPFs. Since the rate of fungal movement through the soil profile is low, most of the available spores are retained within the superficial soil layer and persist within roots and insects after soil application [39,40]. Considering that most *T. absoluta* individuals pupate in the soil at a depth of 1–2 cm [41], there is a high probability that pupae will come into contact with conidia of entomopathogenic fungi if these are applied as drench treatments.

Plastic mulching is globally used in commercial greenhouses to increase plant productivity, conserve soil moisture, modify soil temperatures, and reduce weed pressure [42,43]. These are aspects that warrant further investigation if EPFs are considered as drench treatments. Modification of the soil microclimate caused by mulching could affect the efficacy and persistence of EPFs in the soil [44] and the resulting effects should be considered.

5. Conclusions

The global pest status of *T. absoluta* poses a significant threat to agricultural production and the livelihoods dependant on agricultural sustenance as is the case on the African continent. The default management strategy of applying chemical insecticides to control this pest and subsequent insecticide resistance evolution highlights the importance of using integrated management approaches in which biopesticides such as EPFs play a crucial role. This study provides evidence of the suppressive potential when administering EPF conidia as a soil drench to disrupt the life cycle of *T. absoluta* for the use in integrated pest management programs, preferably in combination with other strategies aimed at control of eggs and larvae. However, further studies need to be performed, focusing on the efficacy and persistence of EPFs as a soil drench under field conditions in soil types with different properties. Furthermore, the effectivity of EPF application via drip irrigation and the efficacy of EPFs when plastic mulching is implemented should also be investigated.

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Data Availability Statement: All data are provided in the paper.

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Chapter 3

Wolbachia strain associated with South African *Tuta absoluta* populations, and the lack of genetic diversity and parthenogenesis

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Abstract: Pheromone-based control strategies centred on mating disruption and mass trapping form an integral part of integrated management approaches of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). However, the ability of *T. absoluta* to reproduce asexually through parthenogenesis, which is believed to be a consequence of infection by the bacterial endosymbiont, *Wolbachia* (Hertig) (Rickettsiales: Ehrlichiaceae), has been speculated as a possible limiting factor to the success of these management strategies. This study aimed to determine the frequency of *Wolbachia* infection and strain composition in South African populations of this pest, to determine the genetic diversity of South African populations and the effect that *Wolbachia* may have on reproduction. The genetic diversity of *Tuta absoluta* samples from four localities in South Africa was investigated by employing a fragment of the mitochondrial cytochrome c oxidase subunit I and the multilocus sequence typing scheme to identify the strain of *Wolbachia* in these populations. Virgin female moths were isolated in parthenogenesis bioassays after which fecundity, fertility and female lifespan was recorded. High rates (≥ 95%) of infection with a single *Wolbachia* strain (ST41) was observed. Genetic diversity was low and consisted of only four haplotypes, one of which was novel. None of the eggs laid by unmated females were viable. We conclude that *Wolbachia* infection in South African *T. absoluta* populations does not lead to either parthenogenesis or failure of pheromone-based strategies.

Keywords: endosymbiont, Gelechiidae, male annihilation, mating disruption, South American tomato pinworm

1 Introduction

The South American tomato pinworm, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), has since the 1950s been considered as one of the most damaging pests of tomato in its native region of South America (Barrientos et al. 1998; Miranda et al. 1998). However, its invasion into Europe in 2006, followed by a spread across the Afro-Eurasian supercontinent changed the pest status of *T. absoluta* and it became a global threat to tomato production (Campos et al. 2015; Carvalho et al. 2018). This pest has the ability to cause yield losses up to 100% if left unmanaged (Chermiti et al. 2009; Desneux et al. 2011).

The South American tomato pinworm is difficult to control, particularly in open-field cultivation systems. Control often requires intensive application of insecticides with several cases of insecticide resistance already reported for this pest (Guedes et al. 2019; Roditakis et al. 2009). It is therefore important that an integrated approach that includes biological control and pheromone-based methods, in combination with synthetic insecticides when needed, is implemented for management of *T. absoluta* (El-Arnaouty et al. 2014; Guedes et al. 2019; Mandour et al. 2012; Senior & McEwen 2001; Shalaby et al. 2013; Zekeya et al. 2019). Since the identification of the species' pheromone blend in the late 1990s, pheromone-based strategies such as male annihilation and mating disruption played important roles in integrated management of this pest (Ferrara et al. 2001; Filho et al. 2000). The pests' amphimictic reproduction and earlier emergence of males compared to females, support the use of male annihilation as a control method (Lee et al. 2014; Tropea Garzia et al. 2012). Nevertheless, male annihilation by means of pheromone baited traps as a single strategy is not effective in reducing leaf and fruit damage, even though large numbers of males are caught (Abbes & Chermiti 2011; Cocco et al. 2012). Inadequate control was also reported in both low-containment greenhouses and open-field production systems with synthetic pheromone treatments applied for mating disruption of *T. absoluta* (Cocco et al. 2012; Filho et al. 2000; Vacas et al. 2011). These failures have in part been ascribed to the ability of *T. absoluta* to reproduce parthenogenetically, a phenomenon first documented in a population collected in France in 2011 (Caparros Megido et al. 2012).

This species' ability to reproduce asexually was later confirmed in Tunisian and Chinese populations of the pest which were able to produce progeny in the absence of males (Abbes & Chermiti 2014; Wang et al. 2021). The ability of *T. absoluta* to undergo deuterotokous parthenogenesis has long been speculated to be a consequence of manipulation by specific strains of the bacterial endosymbiont *Wolbachia* (Rickettsiales: Rickettsiaceae) (Abbes & Chermiti 2014; Caparros Megido et al. 2012).

The maternally inherited endosymbiont *Wolbachia* is estimated to occur in 66% of all insects (Hilgenboecker et al. 2008) with its numerous strains affecting host reproductive biology in diverse ways, including parthenogenesis, cytoplasmic incompatibility, male killing and feminization of genetic males (Biondi et al. 2018; Correa & Ballard 2012; Engelstädter & Telschow 2009). Consequently, attempts were made to exploit the endosymbiont for use in arthropod pest management, mainly through incompatible insect techniques (IIT) (Nikolouli et al. 2018; Saridaki & Bourtzis 2017). However, it is the ability of *Wolbachia* to alter the reproductive biology of its arthropod hosts into parthenogenetic reproduction that is of concern when management tools such as male-annihilation and mating disruption are compromised.

High rates of *Wolbachia* infection (≤95%) were reported in *T. absoluta* populations from both Montenegro and Croatia in Eastern Europe (Škaljac et al. 2012). However, neither of the *Wolbachia* strains associated with these populations were identified, nor was the ability to reproduce parthenogenetically evaluated (Škaljac et al. 2012). Similarly, Carvalho et al. (2018) reported high levels (70 - 100%) of *Wolbachia* infection in Brazilian *T. absoluta* populations. The latter populations were infected with only a single *Wolbachia* strain (ST41), except for one, in which both the phylogenetically closely related ST41 and ST354 strains were found. Females infected with the *Wolbachia* strain (ST41) did not lay any viable eggs (Carvalho et al. 2018), and no link between *Wolbachia* infection and parthenogenesis in Brazilian *T. absoluta* populations was found. Although *T. absoluta* is widespread and has a significant economic impact in South Africa, no information is available on the incidence and possible effects of *Wolbachia* in *T. absoluta* populations.

The objectives of this study were to (i) assess the frequency of *Wolbachia* infection in South African *T. absoluta* populations, (ii) identify the strain compositions of the endosymbiont, (iii) determine the genetic diversity of the host, and finally (iv) to determine whether South African populations of this pest reproduce parthenogenetically.

2 Materials and Methods

2.1 Insect populations and DNA extraction

South American tomato pinworm populations were collected from infested commercial tomato fields at Piet Retief (PR), Mpumalanga province (26°54'23.0"S 30°47'59.1"E), Tarlton (TT), Gauteng province (26°04'08.1"S 27°41'50.2"E), Ventersdorp (VD), North West province (26°23'36.9"S 26°45'52.3"E) and Mooketsi (MK), Limpopo province (23°34'59.5"S 30°08'49.3"E). In the laboratory, infested leaves were placed onto a plastic mesh (hole diameter = 2 cm) which was suspended from the roof of an insect rearing cage (100 cm x 100 cm x 150 cm), above healthy potted tomato plants (cv. Monica). Larvae were thus able to migrate from infested leaves onto potted tomato plants.

Twenty adults of the field-collected specimens per population were sampled and stored at -20˚C for subsequent extraction of genomic DNA. The abdomens of collected individuals were homogenized for DNA extraction. Extractions were done using the DNeasy Blood and tissue kit (Qiagen, Johannesburg, South Africa), according to the manufacturer's protocol.

2.2 *Wolbachia* strains and tomato pinworm populations

The multilocus sequence typing (MLST) scheme was used for detection and determination of the *Wolbachia* strains infecting these populations. The MLST scheme was developed as a universal genotyping tool for *Wolbachia* by indexing the variation of internal fragments of five conserved genes (*gatB, coxA, hcpA, ftsZ* and *fbpA*), to accurately differentiate between strains of the endosymbiont. Additionally, the *Wolbachia* surface protein (WSP, wsp locus) typing scheme was used as a supplemental strain marker. Both the MLST and WSP protocols were performed according to Baldo et al. (2006).

All PCR reactions were performed in a 25 µl volume including 12.5 µl ready to use master mix (Promega Corporation, Madison, United States of America), 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 3 µl DNA and 7.5 µl ddH2O. Amplification of DNA was carried out using a ProFlex 96-well PCR system (Applied Biosystems, Johannesburg, South Africa) under the following thermal profiles: 2 min denaturation at 94˚C, 37 cycles of 30 s denaturation at 94˚C, 45 s annealing at 54˚C for gatB, coxA, hcpA, ftsZ and 59˚C for fbpA and wsp respectively, 1.5 min elongation at 72˚C, and a final extension step of 10 min at 72˚C.

The genetic diversity of South African *T. absoluta* populations was assessed by amplifying the mitochondrial cytochrome c oxidase subunit I (COI) for five individuals per population, using the LCOI490 and HCOI2198 primers (Folmer et al. 1994). PCR reactions for COI were performed as mentioned above at the following thermal profile: 2 min denaturation at 94˚C, 38 cycles of 30 s denaturation at 94˚C, 45 s annealing at 52.5˚C, 1.5 min elongation at 72˚C, followed by a final extension of 10 min at 72˚C.

After DNA amplification was completed, 3 µl of PCR product stained with GelRed (Biotium, Fremont, United States of America) was electrophoresed on 1% agarose gel and visualised using a UV transilluminator to determine the host infection status and quality of DNA fragments. Ten *Wolbachia*-positive samples were randomly selected per population, of which the PCR products were purified and sequenced by Inqaba Biotech™, South Africa (www.inqaba-southafrica.co.za), using the same primers described above, in the amplification step.

2.3 Data construction and phylogenetic studies

DNA sequences obtained from Inqaba Biotech™ were edited using Chromas version 1.45 (McCarthy 1997), after which the *Wolbachia* MLST gene fragments were concatenated to provide a specific identification along with allelic profiles defined for WSP using four hypervariable regions. Concatenated MLST and WSP *Wolbachia* sequences that were used to produce a phylogeny with *Wolbachia* strains were obtained from the MLST *Wolbachia* isolate data bank (PubMLST) (Table S1) and aligned with the ClustalW alignment tool (Larkin et al. 2007), Geneious Prime 2020.2.3 (Kearse et al. 2012).

The COI dataset of *T. absoluta* used for phylogenetics comprised of two sequences obtained in this study and 22 sequences available in the NCBI database (Table S2) that represent worldwide *T. absoluta* distribution. Additionally, 20 sequences obtained in this study and 199 sequences from the NCBI database were used to estimate a series of population-level parameters including pairwise nucleotide diversity (π), haplotype diversity (h) and neutrality tests using DnaSP 5.10.01 (Librado & Rozas 2009).

The most suitable model of nucleotide substitution was determined using the jModelTest 2.1.7 program (Darriba et al. 2012). The GTR+G+I was identified for *Wolbachia* concatenated MLST genes whilst the GTR+G model was most suitable for the *T. absoluta* COI gene. Bayesian inference (BI) was performed using MrBayes 3.2.6 (Huelsenbeck & Ronquist 2001) in Geneious Prime 2020.2.3. Two Monte Carlo Markov chains were run to generate samples from posterior distributions of trees and substitution rate parameters, given the alignments. The chains were run for one million generations with a sample frequency of 1000. Results were summarized in a 50% majority-rule consensus tree.

2.4 Parthenogenesis bioassay

Tuta absoluta pupae from the field-collected larvae (F0), representative of the most common haplotype identified in the phylogenetic study described above were used in the bioassay. Pupae were sexed according to the positioning of genital openings (Coelho & França 1987) and placed individually in plastic containers (height: 5.2 cm, diameter: 3.0 cm). After adult emergence, 60 virgin females were individually transferred into well ventilated oviposition cages (height: 15 cm, diameter: 9.0 cm) and provided with a fresh tomato shoot (cv. Moneymaker) as an oviposition substrate. The shoot was inserted into a plastic tube (height: 5.5 cm, diameter: 1.2 cm), filled with water. Additionally, 60 male-female pairs were kept in oviposition cages and allowed to sexually reproduce under similar conditions as described above. Fecundity and fertility of moths were recorded daily, until the females died. Tomato shoots were replaced daily. The shoots that were removed, were maintained and the viability of the eggs on these shoots, was assessed over a period of 12 days. Containers were maintained at 26 ± 1 ˚C, 65% RH with a 14L: 10D photoperiod.

2.5 Data analysis

Fecundity, fertility and female lifespan data were tested for normality with the Shapiro-Wilk test, and for homogeneity using the Levene's test. Since data were neither normally distributed nor homoscedastic, they were analysed using the Mann-Whitney test.

3 Results

3.1 *Wolbachia* strains and genetic variability of COI sequences

All the populations screened in this study exhibited *Wolbachia* infection. ranging between 95 and 100%. All infected individuals were infected with a single *Wolbachia* strain, identified as ST41 (Table 1). The ST41 strain is classified in the *Wolbachia* supergroup B (Fig. 1).

Characterization of the COI (710-bp) gene placed the four populations evaluated in this study into a single haplotype (GenBank accessions MZ735728, MZ735730-MZ735747). However, one individual from the Ventersdorp (VD) locality (GenBank accession MZ735729), was identified as a novel haplotype (Fig. 2). The worldwide haplotype diversity (Hd) was 0.239, nucleotide diversity (π) was 0.00008 with only four haplotypes identified (Table 2). A value of -0.66086 was reported for Tajima's neutrality test for the worldwide *T. absoluta* COI sequences (Table 2).

Table 1. *Wolbachia* strain allelic composition and the *wsp* surface protein gene associated with *Tuta absoluta* sampled at different localities in South Africa.

Location	**Infection rate (%)**	**ST**	***gatB***	***coxA***	***hcpA***	***ftsZ***	***wsp***	**HVR1**	**HVR2**	**HVR3**	**HVR4**
Mooketsi (MK)	95	41	39	14	40	4	36	10	8	10	8
Piet Retief (PR)	100	41	39	14	40	4	36	10	8	10	8
Tarlton (TT)	100	41	39	14	40	4	36	10	8	10	8
Ventersdorp (VD)	100	41	39	14	40	4	36	10	8	10	8

ST: sequence type.

HVR: hypervariable regions of the *wsp* gene

Table 2. Genetic variability of *Tuta absoluta* COI sequences according to DnaSP.

	South Africa	**Worldwide**
Sample size	20	219
No. of haplotypes (Nh)	2	4
Haplotype diversity (h)	0.100	0.239
Nucleotide diversity (π)	0.00021	0.00008
No. of segregating sites (S)	1	6
Tajima's D	-1.16439	-0.66086

Accession numbers are provided in supplementary table S1

Table 3. Mean number of eggs, female longevity and fertility of virgin and mated *Tuta absoluta* females.

Treatment	***n*** [a]	**Mean fecundity/female (± SE)**	**Mean female lifespan (days ± SE)**	**Mean fertility (% ± SE)**
Mated	60	81.26 ± 7.28	8.0 ± 0.49	94.4 ± 0.88
Unmated	60	5.68 ± 0.98	20.07 ± 0.92	0
		$p<0.001$	$p<0.001$	$p<0.001$

[a] *n* = Number of female moths

3.2 Parthenogenesis bioassay

From the 60 virgin females that were isolated, 43 were able to oviposit. The number of eggs per female ranged from one to 35. The mean fecundity of virgin females (5.68 eggs per female) was significantly lower compared to mated females (81.26 eggs per female) (Table 3). With a lifespan of approximately 20 days, virgin females survived almost three times longer than mated females (8 days) after emergence (Table 3). The fertility rate of mated females was 94.64%, while none of the eggs laid by virgin females were viable (Table 3).

4 Discussion

The *Wolbachia* infection rate in South African *T. absoluta* populations is high (≥ 95%). It is similar to infection rates reported in *T. absoluta* populations from Croatia and Montenegro (Škaljac et al. 2012), which ranged between 95 and 100% and from Brazil which ranged between 70 and 100% (Carvalho et al. 2018). The *Wolbachia* strain, ST41, identified in this study, is the same strain reported in all Brazilian *T. absoluta* populations. *Wolbachia* strain ST41 was phylogenetically sorted in supergroup B, one of the most widely distributed supergroups (along with supergroup A) in the class Insecta, and commonly associated with lepidopterans (Fig. 1) (Ravikumar et al. 2010; Zhou et al. 1998). The low diversity and strong prevalence of a single *Wolbachia* strain in *T. absoluta* along with the lack of diversity in the WSP system, suggest a recent single infection of the host by this endosymbiont (Carvalho et al. 2018). However, a recent re-evaluation of MLST of *Wolbachia* concluded that MLST markers are too conserved to allow for effective fine-scale strain differentiation and argued that whole genome typing approaches should rather be used in the future (Bleidorn & Gerth 2018). The inability to differentiate between *Wolbachia* strains when using MLST has also been reported for *Wolbachia* identified from *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) which was identical at all five loci to the strain found in *Drosophila simulans* (Sturtevant) (Diptera: Drosophilidae), with polymorphisms observed only after conducting genomic sequencing (Siozios et al. 2013). Therefore, low *Wolbachia* diversity in *T. absoluta* could also be assigned to the inability to differentiate between strains when using MLST sequencing with WSP typing.

The predominant COI haplotype found in this study occurs worldwide, including in the pests’ native areas in South America (Carvalho et al. 2018). It differs from the other haplotype present in South Africa (VD) by one segregating site only. Out of the 239 COI sequences of *T. absoluta* available globally, only four haplotypes were identified. These are the predominant haplotype with a worldwide distribution, the novel VD haplotype identified in this study (GenBank accession MZ735729), a haplotype from Kenya (Genbank accession KP324753), and lastly, one haplotype from India (GenBank accession KX467347) (Fig. 2).

The low genetic diversity in South African *T. absoluta* populations is in accordance with reports from South America, Europe, Asia and Africa (Carvalho et al. 2018; Cifuentes et al. 2011; Flores et al. 2003; Shashank et al. 2018). In the case of invasive species, higher heterozygosity is normally observed in its native areas when compared to newly invaded areas, where populations only contain a subset of the genetic diversity present in the original population (Guillemaud et al. 2015; Kliber & Eckert 2005; Young et al. 2018). A reduction in genetic diversity is usually considered to be harmful, but in some cases it may contribute to the success of an invasive species. Cifuentes et al. (2011) concluded that genetic homogeneity in *T. absoluta* populations from the Mediterranean could not be assigned to founder effects, due to the lack of genetic diversity of the pest even in its native distribution range when using internal transcribed spacers (ITS) mitochondrial COI.

However, Guillemaud et al. (2015) reported higher levels of differentiation when they used microsatellite markers to determine the exact source of the invasive population in the Mediterranean basin. A 20% allele frequency variance was reported between the northern and southern regions of South America, the native area of the pest (Guillemaud et al. 2015). A lack of genetic structure was also observed in areas newly invaded by *T. absoluta*, even when microsatellite markers were used. This supports the notion (Shashank et al. 2018) that there was a single introduction of *T. absoluta* into either Spain or Africa followed by an expansion without a demographic bottleneck. This conclusion made by Guillemaud et al. (2015) also explains the negative Tajima’s D value reported in this study, allocating the low frequency of polymorphisms relative to expectation as a result of recent population expansion after a single introduction into newly invaded areas.

Integrated pest management approaches, which include pheromone-based control methods are important for effective control of *T. absoluta* (Biondi et al. 2018; El-Arnaouty et al. 2014; Erasmus et al. 2021; Never et al. 2017; Wang & Wu 2012). However, the ability of female *T. absoluta* to produce viable eggs through tychoparthenogenesis (accidental) in the absence of males, poses a major threat to the effectiveness of pheromone-based strategies such as mass trapping and mating disruption (Abbes & Chermiti 2014; Caparros Megido et al. 2012).

Parthenogenesis has been reported in *T. absoluta* in populations from France, Tunisia and China (Abbes & Chermiti 2014; Caparros Megido et al. 2012). In these cases, parthenogenesis was hypothesized to be the result of either classical automatic tychoparthenogenesis or microbial manipulation by an endosymbiont such as *Wolbachia*. The study by Wang et al. (2021), however, concluded that polygyny in *T. absoluta* is more likely to disrupt pheromone-based management tools than parthenogenesis with a low hatch rate of 1.1%.

A study by Carvalho et al. (2018) attempted to establish a correlation between *Wolbachia* infection and the ability of Brazilian *T. absoluta* populations to reproduce asexually through parthenogenesis. However, this could not be accomplished as none of the virgin females from Brazilian populations were able to produce viable eggs even though 70 to 100% of all individuals screened, were infected with *Wolbachia* strain ST41 (Carvalho et al. 2018). It was therefore speculated that the two reported cases of parthenogenesis were the result of *Wolbachia* infection after its introduction into Europe, which then also spread to Africa. According to the MLST sequencing from this study, South African populations were infected with the same strain (ST41) as Brazilian populations and no parthenogenesis was also found in this study.

Cytoplasmic incompatibility, male feminization and sex-specific lethality are the other possible effects that *Wolbachia* infection can cause in the host species (Werren et al. 2008). In this study, all individuals were infected with a single *Wolbachia* strain, and testing of these alternate *Wolbachia*-induced aberrations was therefore not possible.

Symbiont-induced parthenogenesis could also be affected by the genotypes present in the host population. The symbiont strain or species is however more likely to play a major role in inducing these affects in *T. absoluta*, due to the genetic homogeneity of the insect host (Carvalho et al. 2018; Cifuentes et al. 2011; Shashank et al. 2018).

No parthenogenesis was observed in any of the South African *T. absoluta* populations in this study. The inadequate control reported with pheromone-based control strategies can therefore not be attributed to *Wolbachia*-induced parthenogenesis in South Africa. The high incidence of a single strain *Wolbachia* infection is more commonly associated with cytoplasmic incompatibility, which provides future prospects of using this endosymbiont as a management tool in incompatibility programmes. Future studies should focus on developing protocols for microsatellite markers to more accurately determine genetic diversity in *T. absoluta*. Furthermore, whole genome sequencing is suggested to allow fine-scale differentiation between closely related *Wolbachia* strains.

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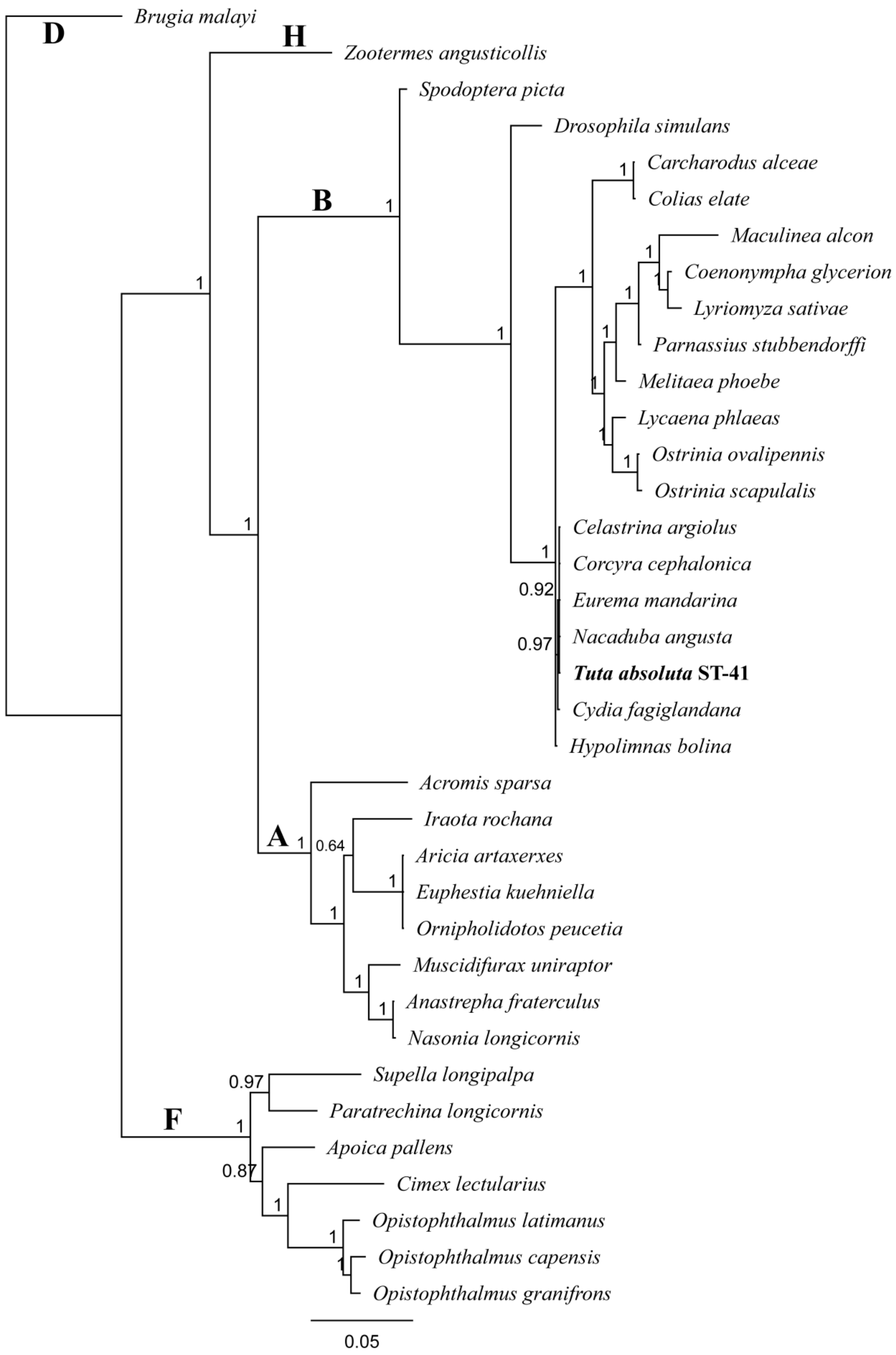
Brugia malayi
D
H
Zootermes angusticollis
Spodoptera picta
Drosophila simulans
Carcharodus alceae
Colias elate
B
Maculinea alcon
Coenonympha glycerion
Lyriomyza sativae
Parnassius stubbendorffi
Melitaea phoebe
Lycaena phlaeas
Ostrinia ovalipennis
Ostrinia scapulalis
Celastrina argiolus
Corcyra cephalonica
Eurema mandarina
Nacaduba angusta
Tuta absoluta ST-41
Cydia fagiglandana
Hypolimnas bolina
Acromis sparsa
Iraota rochana
A
Aricia artaxerxes
Euphestia kuehniella
Ornipholidotos peucetia
Muscidifurax uniraptor
Anastrepha fraterculus
Nasonia longicornis
Supella longipalpa
Paratrechina longicornis
F
Apoica pallens
Cimex lectularius
Opistophthalmus latimanus
Opistophthalmus capensis
Opistophthalmus granifrons
1
0.92
0.97
0.64
0.87
0.05

Fig. 1.

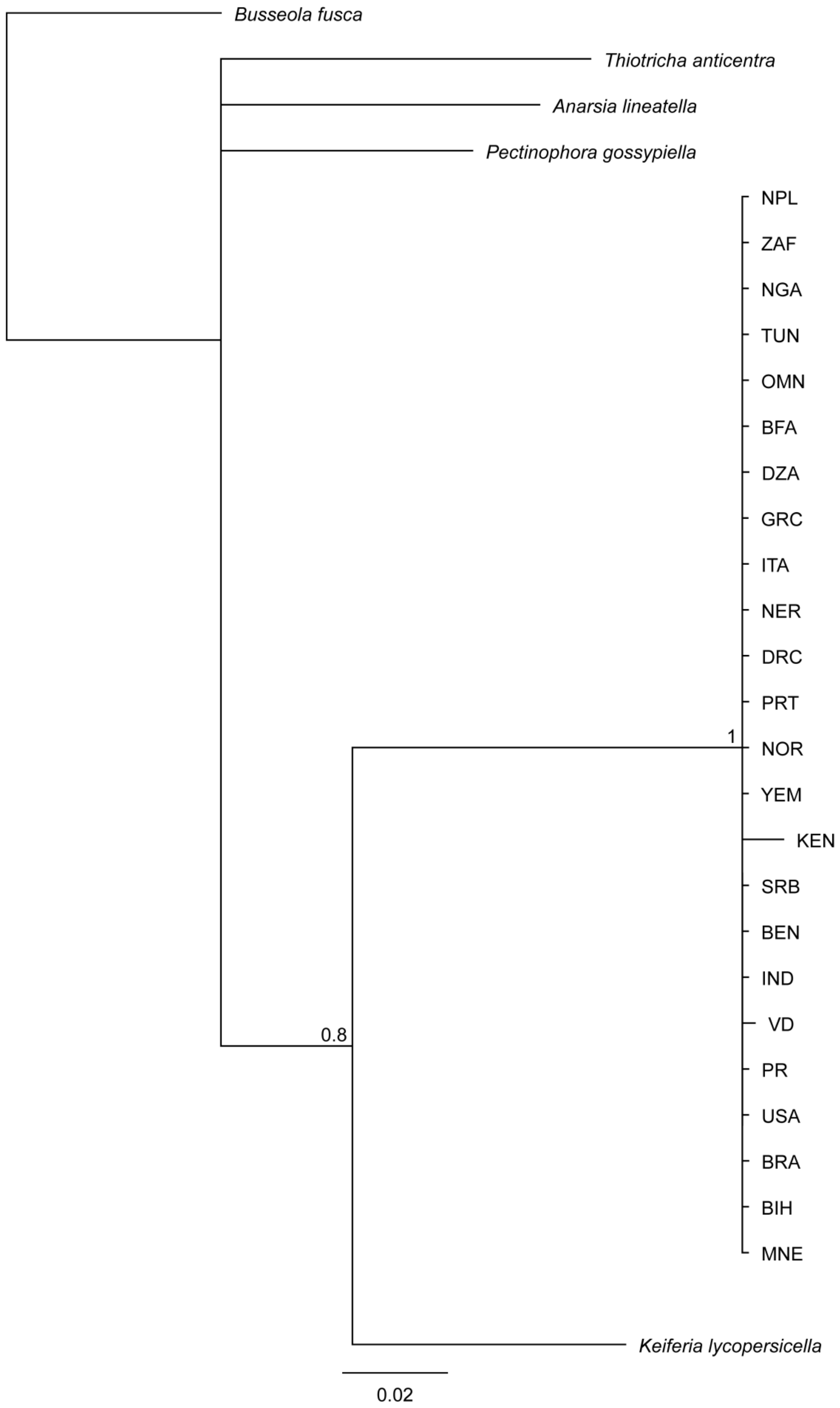
Busseola fusca
Thiotricha anticentra
Anarsia lineatella
Pectinophora gossypiella
NPL
ZAF
NGA
TUN
OMN
BFA
DZA
GRC
ITA
NER
DRC
PRT
1
NOR
YEM
KEN
SRB
BEN
IND
VD
0.8
PR
USA
BRA
BIH
MNE
Keiferia lycopersicella
0.02

Fig. 2

Figure legends

Fig. 1. Bayesian inference (BI) with 50% majority rule of *Wolbachia* strains under the GTR+G+I model. Posterior probabilities more than 50% are provided. Bold letters (A, B, D, F, H) indicate *Wolbachia* supergroups. The *Wolbachia* strain sequence from this study is indicated in bold. (Additional sequence information provided in supplementary table S3).

Fig. 2. Bayesian inference (BI) with 50% majority rule of COI gene sequences of *Tuta absoluta* and other lepidopterans (*Anarsia lineatella*, *Busseola fusca, Keiferia lycopersicella*, *Thiotricha anticentra*, *Pectinophora gossypiella*) as outgroup under the GTR+G model. Posterior probabilities more than 50% are provided. The novel haplotype (VD) and predominant South African haplotype (PR) are indicated in bold. (Accession numbers are provided in supplementary table S1).

Supplementary tables

Supplementary table S1. Additional information (Accession Number, Sampling sites, and reference) for COI sequences of *Tuta absoluta* obtained from NCBI used for phylogenetics.

Accession Number	Sampling sites	Reference
KJ657680.1	United States of America (USA)	Hayden et al. (2014)
KC852872.1	Montenegro (MNE)	Djuric et al. (2013)
KC852871.1	Bosnia (BIH)	Djuric et al. (2013)
JN417242.1	Serbia(SRB)	Tosevski et al. (2012)
KX467347.1	India (IND)	Rakshit et al. (2017)
KT452897.1	Oman (OMN)	Patankar et al. (2016)
KP324753.1	Kenya (KEN)	Tonnang et al. (2015)
JQ749685.1	Tunisia (TUN)	Bettaibi et al. (2012)
KX443110.1	Yemen (YEM)	Sint et al. (2016)
MN066587.1	Niger (NER)	Ndiaye et al. (2021)
MN066585.1	Italy (ITA)	Ndiaye et al. (2021)
MN066584.1	Greece (GRC)	Ndiaye et al. (2021)
MN066582.1	Barkino Faso (BFA)	Ndiaye et al. (2021)
MN066581.1	Algeria (DZA)	Ndiaye et al. (2021)
MK189162.1	Nigeria (NGA)	Bala et al. (2019)
MH882452.1	Republic of Benin (BEN)	Karlsson et al. (2018)
MG693223.1	Democratic Republic of Congo (DRC)	Mukwa et al. (2020)
KY619680.1	Nepal (NPL)	Shashank et al. (2018)
MN804299.1	Norway (NOR)	Huemer et al. (2020)
MN806287.1	Portugal (PRT)	Huemer et al. (2020)
MG596129.1	Brazil (BRA)	Carvalho et al. (2018)

Supplementary table S2. Additional sequences information (Accession Number, Sampling sites, and reference) for COI sequences of *T. absoluta* obtained from the Genbank for building the COI dataset used in DnaSP.

Accession Number	**Sampling sites**	**Reference**
KJ657679.1–KJ657681.1	EUA/flórida	Hayden et al. (2014)
KP814055.1–KP814057.1	India	Shashank et al. (2015)
KC852872.1	Montenegro	Djuric et al. (2013)
KC852871.1	Bosnia	Djuric et al. (2013)
JN417242.1	Serbia	Tosevski et al. (2012)
KP793741.1–KP793742.1	India	Asokan et al. (2015)
KX467347.1	India	Rakshit et al. (2017)
KT452897.1	Oman	Patankar et al. (2016)
KP324752.1–KP324753.1	Kenya	Tonnang et al. (2015)
JQ749676.1–JQ749678.1	Tunisia	Bettaibi et al. (2012)
JQ749680.1–JQ749690.1	Tunisia	Bettaibi et al. (2012)
KX443108.1–KX443111.	Andes	Sint et al. (2016)
MN066591.1	Tunisia	Ndiaye et al. (2021)
MN066586.1–MN066587.1	Niger	Ndiaye et al. (2021)
MN066585.1	Italy	Ndiaye et al. (2021)
MN066584.1	Greece	Ndiaye et al. (2021)
MN066582.1	Barkino Faso	Ndiaye et al. (2021)
MN066580.1–MN066581.1	Algeria	Ndiaye et al. (2021)
MK779005.1	China	Zhang et al. (2020)
MK189155.1–MK189162.1	Nigeria	Bala et al. (2019)
MH882429.1–MH882452.1	Republic of Benin	Karlsson et al. (2018)

MG693216.1–MG693223.1	Democratic Republic of Congo	Mukwa et al. (2020)
KY619673.1–KY619687.1	India	Shashank et al. (2018)
KY923648.1	India	Shashank et al. (2018)
MN804299.1	Norway	Huemer et al. (2020)
MN806287.1	Portugal	Huemer et al. (2020)
KY212128.1	South Africa	Visser et al. (2017)
MG596129–MG596228	Brazil	Carvalho et al. (2018)

Supplementary Table S3. Additional sequence information of *Wolbachia* strains obtained from MLST *Wolbachia* (PubMLST) data bank for building strain phylogeny.

Host species	**Order**	**Sequence typing (ST)**	**Supergroup**
Acromis sparsa	Coleoptera	5	A
Anastrepha fraterculus	Diptera	13	A
Aricia artaxerxes	Lepidoptera	19	A
Ephestia kuehniella	Lepidoptera	19	A
Hypolimnas bolina	Lepidoptera	91	A
Iraota rochana	Lepidoptera	110	A
Muscidifurax uniraptor	Hymenoptera	23	A
Nasonia longicornis	Hymenoptera	24	A
Ornipholidotos peucetia	Lepidoptera	19	A
Carcharodus alceae	Lepidoptera	297	B
Celastrina argiolus	Lepidoptera	41	B
Coenonympha glycerion	Lepidoptera	301	B
Colias elate	Lepidoptera	141	B
Corcyra cephalonica	Lepidoptera	41	B
Cydia fagiglandana	Lepidoptera	150	B
Drosophila simulans	Diptera	15	B
Eurema mandarina	Lepidoptera	41	B
Hypolimnas bolina	Lepidoptera	148	B
Lycaena phlaeas	Lepidoptera	109	B
Liriomyza sativae	Diptera	551	B

Maculinea alcon	Lepidoptera	235	B
Melitaea phoebe	Lepidoptera	302	B
Nacaduba angusta	Lepidoptera	41	B
Ostrinia ovalipennis	Lepidoptera	142	B
Ostrinia scapulalis	Lepidoptera	27	B
Parnassius stubbendorffii	Lepidoptera	299	B
Spodoptera picta	Lepidoptera	579	B
Apoica pallens	Hymenoptera	137	F
Cimex lectularius	Hemiptera	8	F
Opistophthalmus capensis	Scorpiones	62	F
Opistophthalmus granifrons	Scorpiones	72	F
Opistophthalmus latimanus	Scorpiones	64	F
Paratrechina longicornis	Hymenoptera	471	F
Supella longipalpa	Blattodea	456	F
Brugia malayi	Rhabditida	35	D
Zootermes angusticollis	Isoptera	90	H

Chapter 4

Susceptibility of *Tuta absoluta* (Lepidoptera: Gelechiidae) larvae to foliar and systemically applied spinosad on tomato

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Abstract

Tuta absoluta (Lepidoptera: Gelechiidae) is notoriously difficult to control using foliar insecticides, partly due to the endophytic feeding behavior of larvae. Spinosad is a reduced-risk biopesticide administered as a foliar spray for the control of *T. absoluta*. It also has systemic properties when applied as a soil drench to the substrate of tomato plants and therefore holds potential for the control of larvae for a prolonged period of time. However, its efficacy of control, distribution through plants and residual activity has not been reported. This study was carried out to determine (i) the larval instar-dependant tolerance of *T. absoluta* larvae to spinosad (ii) the efficacy of spinosad drench application for the control of *T. absoluta*, (iii) the residual period of the systemic activity of spinosad in leaves and fruit after drenching, and (iv) effect of spinosad drenching on tomato plant growth parameters. The lethal concentrations (LC_{50} and LC_{80}) of spinosad administered as a soil drench were estimated by exposing second-instar larvae to plants drenched with different concentrations of spinosad. Systemic persistence of spinosad in the leaves of tomato plants drenched with this insecticide was determined by means of LC-MS/MS. Larval mortality data were recorded over time after drenching. Although the spinosad concentration in leaves gradually reduced over time, high larval mortalities were recorded for the duration of the experiment, which lasted 25 days. Plant height was positively affected by drench treatment. The levels of spinosad detected in fruit were well below the maximum residual limits set for South Africa. This study provides evidence of the systemic properties of spinosad in tomato and the effectiveness thereof to control *T. absoluta* larvae.

Key words: insecticide drench, insect resistance management, residual period, systemic, toxicity

Introduction

Pesticides use has contributed to the improvement of agricultural production in both quality of produce and yield (Cooper and Dobson 2007, Damalas and Koutroubas 2018). The global market for pesticides was anticipated to reach approximately 61.2 billion US$ in 2017, and 79.3 billion US$ by 2022 (Chen 2018). Most of these pesticides are synthetic chemical insecticides that could pose serious risks to consumers and the environment if used indiscriminately without adhering to the safety regulations and recommended practices (Damalas 2009, Damalas and Koutroubas 2018, Sharma et al. 2019, Negatu et al. 2021).

Biopesticides, which are naturally occurring compounds used as pest management agents. They include microorganisms (bacteria, fungi and viruses), substances produced by plants and certain minerals. Biopesticides comprise only 5% of the global crop protection market (Copping and Menn 2000, Pathma et al. 2021). Amongst these compounds, spinosad, which is considered as one of the most promising options, especially with its acceptance in organic farming (Racke 2006, Biondi et al. 2013, Puinean et al. 2013). Spinosad is a mixture of spinosyns A and D, and is a product of the fermentation of a naturally occurring actinomycete, *Saccharopolyspora spinosa* (Pseudonocardiales: Pseudonocardiaceae) (Thompson et al. 2000). With two modes of action, *viz*. depolarizing nicotinic acetylcholine and γ-aminobutyric acid (GABA) receptor neurons, the target spectrum of spinosad includes species from several insect orders (Coleoptera, Diptera, Hymenoptera, Isoptera, Lepidoptera and Thysanoptera) (Sparks et al. 1995, Bret et al. 1997). The tomato pinworm *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) is among the lepidopterans being controlled by spinosad.

Tuta absoluta is one of the major threats to global tomato production (Urbaneja et al. 2007, Desneux et al. 2010, Pfeiffer et al. 2013, Brévault et al. 2014, Chidege et al. 2016, Campos et al. 2017, Visser et al. 2017, Idriss et al. 2018). The application of synthetic chemical insecticides is the most commonly used practice to control this pest. However, selection pressure caused by the overreliance on insecticides has led to resistance development and subsequent reduced efficacy of insecticides (Roditakis et al. 2009, Guedes and Picanço 2012, Wang and Wu 2012, Biondi et al. 2018). In the case of spinosad, field evolved resistance has been reported in *T. absoluta* populations from Brazil, Chile and the United Kingdom (Reyes et al. 2012, Campos et al. 2014, Grant et al. 2019).

Larval instar-dependant insecticide tolerance was reported in several lepidopteran species, with a decrease in susceptibility to insecticides, in later larval instars (Kim et al. 1998, Cook et al. 2004, Yu 2008). In the case of spinosad, both Ramos et al. (2004) and Khan et al. (2010) reported that younger larvae of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) were more susceptible than older larvae. To date, instar-dependant tolerance of *T. absoluta* to spinosad has not been investigated. To delay insecticide resistance to spinosad in South Africa, it is recommended on the product labels to target eggs and early-instar larvae.

In South Africa, spinosad is registered as a foliar spray only. However, the drenching of systemic insecticides to the roots of plants is sometimes practiced for control of *T. absoluta* on tomato. The systemic properties of spinosad, applied as a drench to the roots of tomato plants was demonstrated by Van Leeuwen et al. (2005) against *Tetranychus urticae* (Koch) (Acari: Tetranychidae) and both *Trialeurodes vaporariorum* (Westwood) (Hemiptera: Aleyrodidae) and *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) (Van Leeuwen et al. 2006).

The concentration of the product inside the leaves of drenched plants as well as the period of persistence is, however, unknown and could pose a serious threat in terms of resistance evolution to this insecticide. The objectives of this study were therefore to investigate the larval instar-dependant tolerance of *T. absoluta* to spinosad and to determine the efficacy of control of a drench application at different concentrations. Furthermore, to determine spinosad concentration inside leaves and fruit, the persistence of systemic toxicity in the leaves, and the effects on tomato plant growth parameters.

2 Materials and methods

2.1 Insect rearing

A tomato pinworm rearing colony was established from a population collected from an infested tomato field at Marble Hall in the Mpumalanga province, South Africa (25°01'47.1"S 29°13'38.1"E). Infested leaves were placed onto a piece of plastic mesh (hole diameter = 2 cm) suspended from the roof of an insect rearing cage. Damage-free potted tomato plants (cv. Moneymaker) were placed underneath the suspended leaves to enable larvae to migrate onto the potted tomato plants and complete their life cycle. Adults were collected daily from these rearing cages, and transferred to oviposition cages with damage-free potted tomato plants for oviposition. Cotton swabs soaked in a 10% sugar solution were provided in Petri dishes at the bottom of each cage, as an energy source. Plants were replaced every second day to ensure that the eggs laid would hatch at approximately the same time. Rearing and oviposition cages were maintained at 26 ± 1 °C, 65% RH, and at a 14 L: 10D photoperiod. Second-instar larvae of the F1- and F2-generations of this population were used in experiments.

2.2 Insecticide

A commercial formulation of the spinosyn, Tracer™ 480 SC (Dow AgroSciences Southern Africa (PTY) LTD., Kemptonpark, Gauteng, South Africa), registered as a foliar spray for control of *T. absoluta* in South Africa, was used. The recommended label dosage rate (mid-range) for spinosad was 144 ppm.

2.3 Leaf dip bioassay

Bioassays were conducted according to the leaf-dip bioassay method (IRAC, susceptibility test method No. 022), with 2nd-, 3rd- and 4th-instar larvae. Morphological differences as specified by Sannino and Espinosa (2010) were used to distinguish between the four larval instars. Preliminary range finding experiments were conducted using serial dilutions of spinosad prepared from a stock solution. Based on the results of the preliminary experiment, a range of concentrations per respective instar was selected to provide at least six points between LC_{20} and LC_{80} of the dose-response curve. In total, 10 concentrations per larval instar were prepared, including the control and a concentration expected to result in 100% larval mortality. All dilutions were prepared with deionized water.

Tomato leaflets were immersed for five seconds in different insecticide dilutions containing 0.2% Triton-X-100 and allowed to air-dry for two hours on a plastic mesh with the abaxial leaf surface facing upwards. Each leaflet was transferred into a well of a 32-well bioassay tray (Frontier: Scientific services, Logan, Utah, USA) with the adaxial leaf surface upward. Prior to leaflet transfer, the bottom of each bioassay well was lined with a thin layer of agar (28 g/L) to ensure leaflet turgidity throughout the bioassay period. *Tuta absoluta* larvae in a specific instar, *viz*. 2nd-, 3rd- or 4th-instar, were carefully removed from the galleries of infested tomato leaves. A single larva was transferred to each well. All wells were sealed with transparent ventilated adhesive lids (Frontier: Scientific services, USA). Each concentration consisted of 32 larvae and was replicated three times. Bioassay trays were maintained in incubators under controlled environment of 26 ± 2 °C, 60–65% RH and 16L: 8D. Larval mortality was recorded after 72 h. Larvae were considered dead if they were unable to make coordinated movements in response to an external stimulus (i.e. gentle probing with a fine paintbrush).

2.4 Systemic toxicity of spinosad at different concentrations

The systemic toxicity of spinosad applied to tomato as a drench to control *T. absoluta* larvae was evaluated in a commercial glasshouse. Tomato seedlings (cv. Moneymaker) were planted in 2-L pots (diameter: 16 cm) filled with a 4: 1 soil: compost mixture, up to 2 cm from the brim. The soil contained 1.3% clay, 78% sand, 1.2% silt, with a pH of 6.49. Commercially available compost was used (Culterra, Johannesburg, South Africa). The experiment consisted of 11 treatments, which were serial dilutions ranging between 1.2 – 48.0 ppm prepared from a stock solution. Deionized water was applied as the control treatment. Each treatment was replicated nine times, and each plant inoculated with 12 larvae served as a replicate.

The experiment commenced two weeks after the seedlings were transplanted into pots. Exactly 200 ml of each spinosad dilution was applied evenly onto the soil surface of the pots to obtain a homogeneous distribution of the compound in the substrate. After the initial spinosad application, the plants were watered (200 ml) every fourth day.

Twelve 2nd-instar larvae were inoculated onto the leaves of each plant, 10 days after treatment (DAT). These larvae were confined individually with clip-on cages, to a leaf area of approximately 3.14 cm^2 per larva. Each clip-on cage consisted of a 5 mm high plastic tube with a diameter of 15 mm. Felt on the bottom brim of the tube sealed the tube to the leaf, without causing damage to the leaf surface. The cage was kept in place on the leaf with a hairpin, while the top of the tube was covered with fine mesh (50 micron) to contain the larvae. Larval mortality was recorded five days after larval inoculation.

Temperature was recorded at 30 min intervals with IButton® wireless data loggers (ColdChain ThermoDynamics, Fairbridge Technologies, Johannesburg, South Africa) for the duration of the experiment. The mean minimum and maximum temperature during the experiment was 23.11 ˚C and 31.61 ˚C respectively, with an overall mean (± SD) of 27.24 (± 1.42) ˚C.

2.5 Toxicity and persistence of spinosad applied systemically

The persistence of a drench treatment was determined by exposing larvae to plant leaves at different time intervals after drenching. These time intervals were 2, 10, 15, 20 and 25 DAT. Each treatment consisted of 12 larvae per plant and was replicated eight times.

The experimental design was similar to the one described above. Potted tomato seedlings were drenched with spinosad at a concentration of 48 ppm and plants of the control treatment with deionized water, at an application volume of 200 ml. This translated into the application of 9.6 mg a.i./plant. Second-instar *T. absoluta* larvae were inoculated onto the treated and untreated plants at different time intervals after drenching. Larval mortality was recorded five days after inoculation of each treatment.

The concentration of spinosad inside the leaves over time was determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Leaf samples (± 1 g) were collected from 10 treated and untreated plants at each larval inoculation interval. Leaves were randomly collected per plant and were representative of both old and new growth. Tomato fruit were sampled at the breaker fruit stage from both treated and untreated plants, approximately 80 DAT. Leaf and fruit samples were frozen and stored at -80°C until extraction. All plants were watered (200 ml) every fourth day, and fertilized fortnightly with 100 ml nutrient solution at a rate of 1g/L (Nutrifeed from Stark Ayres (Pty) Ltd., Johannesburg, South Africa).

2.6 Quantification of spinosad

Reagents and chemicals

Spectrometry-grade acetonitrile and water from Honeywell (Johannesburg, South Africa) were used. Formic acid and Ammonium formate were LC-MS eluent additive grade from Sigma-Aldrich (Merck (Pty) Ltd., Modderfontein, South Africa). Spinosad analytical standards were acquired from Sigma-Aldrich (33706-50MG) and Agilent Technologies (distributor: Chemetrix Export (Pty) Ltd., Johannesburg, South Africa) (PST-3735A1000) as 100 µg/mL solutions in acetonitrile. They were mixed and diluted in acetonitrile to the appropriate concentrations and stored in a fridge at 4 °C.

Extraction

A QuEChERS protocol was followed using Agilent Bond Elut QuEChERS kits (p/n 5982-5550) to provide fast and easy extraction. Dispersive SPE clean-up was performed using Agilent Bond Elut QuEChERS dispersive kits, 15 mL dispersive

SPE tubes (p/n 5982-5056). Sample filtration was performed using Captiva Premium Syringe Filter, nylon membrane, 15 mm, 0.2 µm (p/n 5190-5088) (Lucas 2013).

Analytical equipment

Separation was carried out using an Agilent 1290 Infinity binary pump, Agilent 1290 Infinity High Performance auto-sampler and an Agilent 1290 Infinity thermostated column compartment. The liquid chromatograph was coupled to an Agilent Technologies 6470 triple quadrupole LC-MS/MS with an Agilent Technologies Jet Stream electrospray ionization source. Agilent Technologies MassHunter Workstation Software – Qualitative Analysis (ver. B.03.01) was used for data acquisition and data analysis. Analysis was carried out in positive ionisation in dynamic MRM mode using two transitions per compound.

2.7 Effect of spinosad drench application on plant growth parameters

The possible effects of a spinosad drench treatment on plant growth were investigated in a glasshouse experiment. The experiment consisted of 20 spinosad treated plants and 20 untreated plants in a randomised complete block design. Tomato seedlings were planted and maintained in similar conditions as mentioned above. Potted seedlings were treated with spinosad at a rate 48 ppm, with 200 ml solution added per plant. Deionized water was used for control plants.

Plant height, stem diameter, and the fresh mass of whole plants, aerial parts and roots were recorded 30 DAT. Plant height was recorded as the distance from the soil level to the shoot tip along the stem, using a non-stretchable rope to accommodate for stem curvature. Stem diameter was consistently recorded for all plants at 2 cm above the soil level. Total plant root systems were carefully removed from the substrate, rinsed to remove all debris and allowed to air dry for 5 min before recording whole plant, aerial and root mass.

2.8 Data analyses

Abbott's formula was used to correct for larval mortality in the control treatment (Abbott 1925) before estimation of the instar-dependant tolerance of *T. absoluta* larvae to spinosad, and the evaluation of the systemic toxicity of spinosad. Corrected mortality data for leaf dip bioassays and systemic toxicity were subjected to probit analysis and the relative potency ratio among responses was calculated using PoloSuite® software (LeOra Software LLC, version 1.8; Northampton Northamptonshire NN1 2JL, UK). Responses were considered to be significantly different when the 95% confidence interval of the relative potency ratio did not include the value 1.

Data on the residual persistence of spinosad at different concentrations, as well as mortality data over time, were tested for normality (Shapiro-Wilk tests) and homogeneity of variance (Levene's test). Data on the concentration of residual spinosad in the leaves met these assumptions and were subsequently subjected to one-way analyses of variance (ANOVA). Treatment means were separated using the Unequal N Tukey's HSD-test at $p = 0.05$. Corrected percentage mortality data were neither normally distributed nor homoscedastic. It was therefore analysed by means of the non-parametric Kruskal-Wallis test, followed by Dunn's multiple comparison post hoc test. Student t-tests were used to compare the means of the respective plant growth parameters. Analyses were done using TIBCO Statistica™ 13.3 (TIBCO Software Inc. 2017, Palo Alto, CA, USA).

3 Results

3.1 Leaf dip bioassays

Responses of *T. absoluta* larvae from the respective instars fitted the log (dose)/probit (mortality) model at $p < 0.05$ (Table 1). The slope coefficients were similar for both the 2nd- (2.28) and 3rd-instar (2.23) larvae, suggesting a homogenous response to spinosad for these instars. The slope coefficient for 4th-instar larvae (1.42) was considerably lower.

The estimated LC_{50} values of the respective larval instars differed significantly according to the 95% confidence intervals of the relative potency ratio ($p < 0.05$) (Table 1). The estimated LC_{50} value for 2nd-instar larvae (0.41 ppm) was significantly lower than that for both 3rd- (0.64 ppm) and 4th-instar (0.63 ppm) larvae, with no differences in LC_{50} of larvae from the latter two instars. However, the LC_{80} values of 2nd-, 3rd- and 4th-instar larvae differed significantly (Table 1). The LC_{80} value for 4th-instar (2.48 ppm) larvae was 2.6- and 1.7-fold higher than that for the 2nd- and 3rd-instar larvae, respectively.

3.2 Systemic toxicity of spinosad at different concentrations

Mortality of *T. absoluta* larvae that fed on spinosad drenched plants fitted the log (dose)/probit (mortality) model at $p < 0.05$, with a slope coefficient of 2.54 (Table 2). The estimated LC_{50} and LC_{80} values, converted to dosage active ingredient per plant, were 9.35 ppm (1.87 mg a.i./plant) and 20.10 ppm (4.02 mg a.i./plant) respectively (Table 1), with 100% larval mortality at 9.60 mg a.i./plant.

3.3 Residual persistence toxicity of spinosad applied systemically

Good linearity was recorded for spinosyn A (0.01–5.0 ug/g) and spinosyn D (0.01–5.0 0 ug/g) in pure solvent with high correlation coefficients (r^2) > 0.99. Matrix effects were negligible with slope ratios 0.99 for spinosyn A and 1.01 for spinosyn D. The concentration of spinosad present in leaf tissue started to decrease 15 DAT (F = 6.64; df = 4, 42; $p < 0.05$) (Table 3). The spinosad concentration recorded in the leaves at 25 DAT (0.26 µg/g), was significantly lower compared to leaves sampled at 3 – 15 DAT (Table 3). However, although the spinosad concentration decreased, high larval mortalities (96.55 – 100%) were recorded during the entire experimental period (H(4) = 6.69; p = 0.15). Affected larvae stopped feeding within 24 h. The spinosad concentrations in fruit sampled from treated plants 80 DAT were below the detection limit of 0.003 µg/g.

3.4 Plant growth parameters

Plants drenched with spinosad were significantly taller than untreated plants (Table 4) (*t* (38) = 2.75, $p < 0.05$). The other plant growth parameters (stem diameter, and the fresh mass of whole plants, aerial parts and roots) did not differ significantly between the spinosad treated and untreated control plants.

4. Discussion

Results from the leaf dip bioassay indicated instar-dependant tolerance of *T. absoluta* larvae to spinosad. Later-instar larvae (3rd and 4th-instar) were more tolerant to spinosad compared to 2nd-instars. Several aspects of an insects' biology could provide explanations for increased tolerance at later larval instars. Weight increase plays an important role in lethal dose stage dependency (Germano and

Picollo 2018). However, the assumption of a linear relationship between lethal dose and weight could result in an overestimation of lethal dosage in high-weight individuals, and an underestimation in low-weight individuals (Savin et al. 1982). For example, Robertson et al. (1981), reported the lethal doses of insecticides were not directly proportional to different weight groups of 4th-instar *Choristoneura freemani* (Razowski) (Lepidoptera: Tortricidae) larvae.

In addition to weight increase, there are other contributing factors such as degradative metabolism (Germano and Picollo 2018). Kim et al. (1998) attributed the larval instar-dependant tolerance in *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) to enhanced detoxification enzymes and reduced acetylcholinesterase (AChE) activity in later larval instars. Although no significant correlations were found between the reduced susceptibility of spinosad resistant *T. absoluta* larvae in Chile and the activity of glutathione-S-transferases (GST), esterases (EST) and mixed-function oxidases (MFO), enhanced MFO activity was reported as the possible resistance mechanism (Reyes et al. 2012). This resistance mechanism has already been associated with resistance to spinosad in *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) and *S. exigua* (Wang et al. 2006, Wang et al. 2009).

Results on susceptibility of *T. absoluta* from leaf dip bioassays suggest that all larval instars should be effectively controlled by the recommended label rate (mid-range) of 144 ppm. This may explain the current effective control of *T. absoluta* with spinosad in South Africa. The estimated LC_{50} values for 2nd-instar larvae (0.41 ppm) from this study was similar to that of a Brazilian (Campos et al. 2014). The latter population was able to develop spinosad resistance in laboratory trials rapidly, reaching resistance levels of 180 000-fold after only seven generations were subjected to selection pressure (Campos et al. 2014). To date, only three cases of field evolved resistance against spinosad in *T. absoluta* have been reported (Reyes et al. 2012, Campos et al. 2014, Grant et al. 2019).

High mortality of 2nd-instar *T. absoluta* larvae was caused by the systemic toxicity of spinosad administered as a drench. A mortality rate of 80% (LC_{80}) was estimated with drenching with approximately 4 mg a.i./plant (20.1 ppm).

The two most important properties that regulate translocation of pesticides are lipophilicity (log P_{oct} = octanol-water partition coefficient) and acidity (pKa = acid dissociation constant) (Inoue et al. 1998, Vryzas 2016). A study by Inoue et al. (1998) on the physiochemical factors that affect the systemicity of compounds in barley, concluded that compounds with intermediate lipophilicity (log P_{oct} 2 – 3) and acidity (pKa 7.5 to 8.5) are particularly effective in systemic uptake. The log P_{oct} of spinosyn A, the main component of spinosad, ranges between 2.8 and 5.2 with a pKa of 8.10, resulting in effective take-up of spinosyn A by plants (Van Leeuwen et al. 2005).

The systemic persistence of spinosad at levels toxic to 2nd-instar larvae of *T. absoluta*, was at least 25 days, causing 96.51% mortality when drenched at an application rate of 48 ppm (9.6 mg a.i./plant) per plant. This is in contrast to foliar applications of spinosad, where spinosyn A and D rapidly dissipate from foliage predominantly through photolysis. Spinosad has a half-life of 1.6 – 16 days on plant foliage (Thompson et al. 2000). This half-life on foliage is in agreement with the decrease of up to 97% in spinosad residues 10 DAT (spray) on cabbage, cauliflower and okra leaves (Azab 2006, Sharma et al. 2007).

The long systemic persistence of spinosad when applied as a drench is most likely due to the continuous uptake through the root system. A study in which spinosyn A and D were applied to a layer of silt soil, reported quick degradation in the initial stages with half-lives of 17 and 7 days for spinosyn A and D, respectively (Kollman 2002). However, subsequent degradation occurred at a much slower rate with half-lives estimated to exceed 100 days, indicating that some of the residues are absorbed into the soil before UV exposure can take place (Kollman 2002).

The root uptake of spinosad applied as a soil drench to tomato was reported to be much higher in a rockwool substrate, compared to other substrates such as black earth, sand, sand-clay- and peat-clay mixtures (Van Leeuwen et al. 2005, Van Leeuwen et al. 2006). A difference in control of *T. urticae* of approximately 40% was reported when spinosad was systemically applied to rockwool compared to an application to a sandy soil. This can be explained by the higher rate of bacterial breakdown of spinosyns by microbial communities in living soils (Van Leeuwen et al. 2005). Lower spinosad lipophilicity correlates with lower pH levels and therefore lower uptake by partitioning onto root solids. The sandy soil-compost mixture in the current study had a pH of 6.49, lower than the high pH of rockwool, which ranged between 7 – 8 (Van Leeuwen et al. 2005). Since strong sorption of spinosad also occurs in fine-textured soil (Thompson et al. 2000), sandy soils may also be suited to induce systemic toxicity when spinosad is applied as a drench.

The recommended foliar application rates of formulated spinosad to tomato in South Africa range between 72 – 180 g/ha. With an estimated plant density of 20 000 plants/ha in greenhouses, the current registered foliar application rates when applied directly onto these plants range between 3.6 – 9 mg a.i./plant. The estimated LC_{80} for *T. absoluta* when spinosad is administered by means of a drench application is 4.02 mg a.i./plant. This indicates that a drench application rate of 9 mg a.i./plant, similar to the current registered foliar application rate, will provide effective control of *T. absoluta* larvae for at least 25 DAT.

The levels of spinosad detected in tomato fruit (0.003 µg/g) 80 days after drenching with spinosad, were well below the maximum residual limits set for South Africa (0.2 µg/g) and the European Union (0.7 µg/g). In this study, spinosad was drenched during the seedling stage. Considering the persistence of spinosad at high concentrations in leaves 25 DAT, further studies are needed regarding residual limits associated with drenching of spinosad at later plant growth stages, closer to harvest.

Stem diameter, root mass, aerial mass and whole plant mass of tomato plants were not affected by spinosad drenched during the seedling stage. The spinosad treated plants did, however, grow taller compared to the control plants. Thus, spinosad applied as a drench to tomato seedlings had no negative effect on tomato plant growth.

Insecticides exert a selection pressure that favours the survival of resistant genotypes, eventually leading to reduced efficacy as a result of resistance evolution (Roditakis et al. 2009, Guedes and Picanço 2012, Wang and Wu 2012, Biondi et al. 2018). Repeated application of a single insecticidal mode of action (MoA) (Helps et al. 2020) or application of persistent insecticides, as in the case of systemically applied spinosad, would increase the selection pressure due to increased exposure time (Van Leeuwen et al. 2005, Van Leeuwen et al. 2006).

However, insecticide resistance does not only evolve because of the repeated application of a lethal concentration that eliminates all susceptible individuals, but it also favours survival and reproduction of resistant individuals exposed to sub-lethal concentrations (Guedes et al. 2017). Sub-lethal insecticide exposure might delay the selection of major single-gene resistant alleles while favouring the polygenic resistance which results in small increases in the magnitude of insecticide resistance (Gressel 2011). Exposure to sub-lethal insecticide dosages could also influence resistance beyond the selection of resistant individuals through the induction of detoxifying enzymes (Guedes et al. 2017). Inducing detoxification enzymes through sub-lethal insecticide exposure of a population to an insecticide may prime the target insect against the same or other compounds (Costantini et al. 2014, Rix et al. 2015). The continuous decrease in the concentration of spinosad within tomato plants coupled with generational overlapping and larval instar-dependant tolerance could therefore also result in inadequate control and resistance evolution as a result of sub-lethal exposure.

It is, however, not only *T. absoluta*, but the entire species complex feeding on these tomato plants that will be subjected to selection for insecticide resistance. Effective control of more than one pest species could therefore be jeopardized in future by drenching tomato seedlings with spinosad for control of *T. absoluta*. Krechemer and Foerster (2015) reported that *T. absoluta* has an average developmental time of approximately 24 days at 25˚C. Spinosad applied systemically at a rate of 9.6 mg a.i./plant could effectively control *T. absoluta* for at least 25 DAT, thus providing sufficient control for the duration of an entire life-cycle of the target pest. An insecticide with a different MoA should therefore be applied at approximately 20 – 25 DAT. This will ensure that larvae of the follow-up generation are exposed to another MoA, for effective insect resistance management.

Systemic insecticides contaminate all plant tissues (Lue et al. 1984, Meher et al. 2010) including the pollen and nectar of flowers which could have negative effects on pollinators (Sanchez-Bayo et al. 2013). Van Leeuwen et al. (2006) did, however, report that the non-target parasitic wasp, *Encarsia formosa* (Gahan) (Hymenoptera: Aphelinidae), was not affected when exposed to tomato plants drenched with spinosad. Parasitoids could, however, be affected by feeding on hosts that consumed plant material contaminated with the active ingredient (secondary poisoning) (Sanchez-Bayo et al. 2013). Parasitoids may also be indirectly affected by the decrease in host population levels as a result of the long persistence of spinosad (Cloyd and Bethke 2011).

The current study reports on the larval instar-dependant tolerance of *T. absoluta* to spinosad, the systemic properties of spinosad in tomato as well as the effect of systemically applied spinosad on mortality of *T. absoluta* up to 4 weeks after treatment. The levels of susceptibility of 2nd- to 4th-instar larvae estimated in this study, indicated that spinosad applied as a spray treatment at the recommended dose will effectively control *T. absoluta* larvae, regardless of their developmental stage. Further extensive studies need to be performed, focusing on the effect of continuous exposure of pest complexes to residual spinosad present in plant tissue.

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Table 1. Log-dose probit mortality data for different *Tuta absoluta* larval instars treated with spinosad.

Instar	*n* [a] (df)	LC_{50} (ppm)	FL (95%)[b]	LC_{80} (ppm)	FL (95%)	Slope	SE	X^2 [c]
Second	672 (5)	0.41	0.354–0.463 a	0.97	0.842–1.163 a	2.23	1.78	3.51
Third	794 (6)	0.64	0.570–0.705 b	1.49	1.335–1.701 b	2.28	1.17	3.54
Fourth	818 (6)	0.63	0.467–0.801 b	2.48	1.956–3.406 c	1.42	0.12	7.25

Table 2. Log-dose probit mortality for *Tuta absoluta* that fed on tomato plants with systemic spinosad toxicity.

Treatment	*n* [a]	LC_{50} (ppm)	FL (95%)	LC_{80} (ppm)	FL (95%)	Slope	SE	X^2 [b]
Spinosad	640	9.35	8–11.05	20.1	16.45–25.9	2.54	0.17	7.30

[a] *n* = number of larvae tested. [b] Chi-square test for linearity of the dose–mortality response.

Table 3. Mean spinosad concentrations (μg/g ±SE) in tomato leaves over time after drenching with 48 ppm spinosad per seedling and the corrected percentage larval mortality (±SE) on treated plants.

Days after treatment	***n*** [a]	**Mean concentration spinosad (ug/g)** [c]	***n*** [b]	**Mean percentage mortality** [d]
3	9	0.50 ± 0.06 a	96	98.85 ± 1.15 a
10	9	0.46 ± 0.05 a	96	98.85 ± 1.15 a
15	10	0.46 ± 0.03 a	96	100 ± 0.00 a
20	10	0.37 ± 0.03 ab	96	100 ± 0.00 a
25	10	0.26 ± 0.02 b	96	96.55 ± 1.68 a
		$F_{(4,42)} = 6.64$; $p < 0.05$		H(4) = 6.69, $p = 0.15$

[a] *n* = Number of plants sampled. [b] *n* = Number of larvae inoculated. [c] Mean residual spinosad concentration in the leaves at the respective time intervals, provided within the column followed by the same letter, are not significantly different at $p < 0.05$ (Unequal HSD). [d] Mean percentage mortality within the column, followed by the same letter is not significantly different at $p < 0.05$ (Kruskal-Wallis followed by Dunn's multiple comparison test).

Table 4. Comparison of plant growth parameters of spinosad treated and untreated plants.

Treatment	***n*** [a]	**Plant height (cm ± SE)**	**Stem diameter (cm ± SE)**	**Root mass (g ± SE)**	**Aerial mass (g ± SE)**	**Whole plant mass (g ± SE)**
Spinosad	20	40.29 ± 1.36 a	1.34 ± 0.03 a	4.90 ± 0.34 a	15.98 ± 0.87 a	20.88 ± 0.95 a
Control	20	35.49 ± 1.09 b	1.32 ± 0.02 a	4.49 ± 0.2 a	16.19 ± 0.70 a	20.68 ± 0.82 a
		$t(38) = 2.75$ $p < 0.05$	$t(38) = 0.41$ $p = 0.69$	$t(38) = 1.04$ $p = 0.31$	$t(38) = -0.19$ $p = 0.85$	$t(38) = 0.16$ $p = 0.87$

[a] *n* = number of plants. Means within the same column followed by the same letter are not significantly different at $p < 0.05$ (Student's t-test).

Chapter 5

General Discussion and Conclusions

Management of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) is mainly done by means of extensive applications of synthetic insecticides (Arora, 2014; Jaronski, 2010). Unfortunately, insecticide applications are not as effective as expected due to the cryptic feeding behaviour of larvae and the ability of the pest to rapidly develop insecticide resistance (Campos *et al.*, 2014; Lietti *et al.*, 2005; Roditakis *et al.*, 2015). Hence, the integration of synthetic insecticides with more environmentally friendly options, including pheromone-based strategies and biopesticides, becomes highly imperative (Biondi *et al.*, 2018; Guedes & Picanço, 2012; Wang & Wu, 2012).

5.1 Entomopathogenic fungi in biological control

The application of microbial-based biopesticides has been regarded as an effective and environmentally safe alternative control measure for several insect species (Chandler *et al.*, 2011; Czaja *et al.*, 2015; Steven & Surendra, 2019; Van Driesche *et al.*, 2008), including *T. absoluta* (Alikhani *et al.*, 2019; Klieber & Reineke, 2016; Tadele & Emana, 2017).

The use of entomopathogenic fungi (EPFs) represents one of the most promising components of biological control (Sujeetha & Sahayaraj, 2014). Under natural conditions, some EPFs are common and often induce epizootics that regulate insect populations (Sujeetha & Sahayaraj, 2014). Most of the EPF-based biopesticides used worldwide are based on species belonging to the genera *Beauveria* (Hypocreales: Cordycipitaceae)*, Hirsutella* (Hypocreales: Ophiocordycipitaceae)*, Isaria* (Hypocreales: Cordycipitaceae)*, Lecanicillium* (Hypocreales: Cordycipitaceae) and *Metarhizium* (Hypocreales: Clavicipitaceae) (Castro *et al.*, 2016; Khan *et al.*, 2012. With their global distribution, wide host range and vast ecological niches it is no surprise that by far most of the commercially available EPF products are based on *B. bassiana* and *M. anisopliae* (McGuire & Northfield, 2020; Sujeetha & Sahayaraj, 2014).

5.1.1 *Beauveria bassiana* and *Metarhizium anisopliae* for control of *Tuta absoluta*

Beauveria bassiana and *M. anisopliae* have been reported to be pathogenic against *T. absoluta*. Abdel-Raheem *et al.* (2015) reported 100% mortality of *T. absoluta* eggs that were exposed to *B. bassiana* and *M. anisopliae* at a rate of 1×10^7 conidia mL^{-1}. While, İnanlı *et al.* (2012), who used the leaf immersion method to test EPF suspensions against both larvae and eggs, reported egg mortality exceeding 90% after exposure to *M. anisopliae*, but only 67% when treated with a *B. bassiana* product. The larval phase of *T. absoluta* is also highly susceptible to isolates of *B. bassiana* and *M. anisopliae*, with mortalities exceeding 90% after exposure (Hammad *et al.*, 2021; Rodríguez *et al.*, 2006; Tadele & Emana, 2017).

However, studies regarding the susceptibility of *T. absoluta* to EPFs focused largely on their efficacy on the egg and larval stages, with little to no research on the pupal stage. It is important that all life stages be targeted to effectively control *T. absoluta* (Erasmus *et al.*, 2021).

To the best of our knowledge only two studies have evaluated the efficacy of fungal pathogens against *T. absoluta* pupae in the soil and both reported effective control (Contreras *et al.*, 2014; Erasmus *et al.*, 2021). Contreras *et al.* (2014) evaluated a liquid formulation of *M. anisopliae* against *T. absoluta* pupae in Spain and Erasmus *et al.* (2021) (Chapter 2) evaluated an emulsifiable suspension (ES) and a wettable powder (WP) of both *B. bassiana* and *M. anisopliae* in South Africa. When converting the estimated LC_{50} values from laboratory bioassays to conidia/cm^2 the dosages for Spanish populations ranged between 1.17 and 3.0×10^6 conidia/cm^2 (Contreras *et al.*, 2014). All four products screened in laboratory bioassays against pupae from the South African population differed significantly (Chapter 2), with the emulsifiable suspensions (*B. bassiana*: 4.86×10^5 conidia/cm^2; *M. anisopliae*: 3.59×10^6 conidia/cm^2) of both fungal species outperforming wettable powder formulations (*M. anisopliae*: 9.98×10^7 conidia/cm^2; *B. bassiana*: 3.88×10^7 conidia/cm^2) (Erasmus *et al.*, 2021). High pupal mortality (68–98.8%) occurred where EPF products were applied at their respective LC_{80} rates under growth tunnel conditions (Erasmus *et al.*, 2021).

Exposure of the pupal stage to EPFs also negatively impacted female fecundity. *Beauveria bassiana* exposed females laid significantly fewer eggs regardless of male EPF exposure. A reduction in fecundity was also demonstrated with *M. anisopliae*, however only when both males and females were infected (Erasmus *et al.*, 2021).

Soil serves as the natural ecosystem for EPFs, providing fungi with optimal moisture and temperature conditions and protection against UV radiation (Jaronski, 2010). The persistence of fungal pathogens was reported to be higher in the soil than in the phyllosphere, demonstrating that they can naturally favour the control of subterranean pests (Sahayaraj & Namachivayam, 2011). The occurrence and distribution of EPFs in the soil is directly influenced by different soil factors (Qayym *et al.*, 2021). High organic matter, the heaviness of soil texture and soils good in acidity result in harbouring higher percentages of EPFs (Quesada-Moraga *et al.*, 2007). Since the rate of fungal movement through the soil profile is low, most of the available spores are retained within the superficial soil layer and persist within roots and insects after soil application (Garrido-Jurado *et al.*, 2011; Ignoffo *et al.*, 1977). Considering that most *T. absoluta* individuals pupate in the soil at a depth of 1–2 cm (Uchoa-Fernandes *et al.*, 1995), there is a high probability that pupae will come into contact with conidia of entomopathogenic fungi if these are applied as drench treatments. However, the interactions between EPFs and environmental variables, such as temperature, moisture, ultraviolet light exposure and soil characteristics should be investigated (Jaronski, 2010).

5.1.2 Entomopathogenic fungi used in combination with predators

The integration of predatory biological control agents and fungal pathogens can improve the management of *T. absoluta* (Alikhani *et al.*, 2019). Although the wide host range of *B. bassiana* and *M. anisopliae* is a sought after trait for use in EPF products, it can also lead to these fungi infecting non-target natural enemies (Goettel *et al.*, 1990). However, the application of multiple biological control agents may act antagonistically, synergistically or additively (Roy & Pell, 2000). The interactions between predators, parasitoids and fungi are usually beneficial toward EPFs (Brodeur & Rosenheim, 2000). Consequently, in order to make biological control programmes more effective, the interactions (multitrophic) between hosts, crop plants and natural enemies need to be assessed (Alikhani *et al.*, 2019).

Labbé *et al.* (2009) reported that the use of *B. bassiana* could successfully be combined with both the predator *Dicyphus hesperus* (Knight) (Hemiptera: Miridae) and parasitoid *Encarsia formosa* (Gahan) (Hymenoptera: Aphelinidae) for the control of *Trialeurodes vaporariorum* (Westwood) (Hemiptera: Aleyrodidae). Similarly, a study by Thungrabeab and Tonga (2007) reported that *B. bassiana* was non-pathogenic to natural enemies screened, *viz*. *Coccinella septempunctata* (L). (Coleoptera: Coccinellidae), *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) and *Dicyphus tamaninii* (Wagner) (Hemiptera: Miridae). *Metarhizium anisopliae* showed pathogenicity to *C. carnea* and *D. tamaninii*, with low mortalities caused, at 4 and 10%, respectively (Thungrabeab & Tonga, 2007). Conversely, Scorsetti *et al.* (2017) concluded that the population parameters of the predator *Eriopis connexa* (Germar) (Coleoptera: Coccinellidae) were negatively influenced by *B. bassiana*.

The two predatory mirid species commercially available for the control of *T. absoluta* are *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae) and *Macrolophus pygmaeus* (Rambur) (Hemiptera: Miridae) (Desneux *et al.*, 2021). When *N. tenuis* nymphs were sprayed directly with *B. bassiana* or exposed to *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) treated with *B. bassiana,* the effect on nymph survival was low and authors proposed for the two biological agents to be used in combination as part of an integrated pest management (IPM) program (Assadi *et al.*, 2021). In contrast, Nakaishi and Arakawa (2011) reported that *B. bassiana* was extremely harmful to adults and nymphs of *N. tenuis*, resulting in mortality rates of 100 and 97.4%, respectively. Contradictory results and limited quantitative population studies on multispecies interactions between fungal pathogens, predatory mirids and *T. absoluta* currently complicates the understanding of the compatibility of these biological control agents in an IPM program.

5.1.3 Entomopathogenic fungi used in combination with spinosad

Since synthetic insecticides are the preferred method for *T. absoluta* control, the use of EPFs in the field therefore requires an understanding of the impact of insecticides on different fungal pathogens. For example, chloro-organic and organophosphate insecticides exhibited harmful effects on *B. bassiana*, resulting in growth inhibition of between 95 and 100% (Amutha *et al.*, 2010).

However, natural insecticides such as spinosad are far less toxic to EPFs. Asi *et al.* (2010) reported that spinosad caused a reduction of only 20% in conidial germination and 7 – 8% reduction in mycelial growth. Pires *et al.* (2010) also reported the compatibility of spinosad with two *M. anisopliae* isolates. In some cases, the combination of spinosad with EPFs may result in synergistic mortality of the target pest. This was the case with low-dose applications of spinosad preceding *B. bassiana* application, which resulted in a 34% increase in *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) larval mortality when compared to a treatment with only spinosad (Rivero-Borja *et al.*, 2018). Combined treatment with spinosad and *M. anisopliae* as soil drenches for the control of *Agriotes lineatus* (L.) (Coleoptera: Elateridae) and *Agriotes obscurus* (L.) (Coleoptera: Elateridae) resulted in synergistic mortality, which suggests that low-levels of reduced risk pesticides such as spinosad can be combined efficiently for the control of pests with a subterranean life-stage (Ericsson *et al.*, 2007).

5.2 Pheromone-based control strategies of *Tuta absoluta*

Due to its important role in insect mating behavior, the use of sex pheromones could play an important role in the control of agricultural pest species (Desneux *et al.*, 2021). Initially, sex pheromones were only used to detect new infestations of a pest or to monitor the size of an existent pest population (Desneux *et al.*, 2021). Sex pheromones are also used to successfully manage many insect pest species through mating disruption and mass trapping techniques (Cardé & Minks, 1995; Cocco *et al.*, 2012; Witzgall *et al.*, 2010). As in the case of other biological based pest management tools, pheromone-mediated control methods are harmless to the environment, non-target pest species and humans and are suitable to apply in conjunction with biological control methods (Cardé & Minks, 1995; Miller & Gut, 2015; Saha & Chandran, 2017). Pheromone-mediated control strategies are considered promising components of IPM programs (Wang *et al.*, 2021).

Since the identification and synthesis of the sex pheromone blend of *T. absoluta*, male annihilation and mating disruption have played an integral role in the integrated management of this pest (Ferrara *et al.*, 2001; Filho *et al.*, 2000). Mating disruption aims to saturate the crop area with synthetic pheromones to disorientate males in pursuit of a viable mate (Cocco *et al.*, 2013; Witzgall *et al.*, 2010), while the male annihilation technique specifically reduces the number of males available by attracting and catching them in deployed sex pheromone traps (Witzgall *et al.*, 2010). The pests' amphimictic reproduction and earlier emergence of males compared to females support these strategies (Ferrara *et al.*, 2001; Filho *et al.*, 2000).

5.2.1 Control failure of pheromone-based strategies and parthenogenesis

Unsatisfactory control was reported for both male annihilation and mating disruption on several occasions (Abbes & Chermiti, 2011; Cocco *et al.*, 2012; Filho *et al.*, 2000; Vacas *et al.*, 2011). These failures were ascribed to the insufficient density (traps/ha) at which these pheromone traps were deployed or the inability of traps to catch all males in a population (Caparros Megido *et al.*, 2013). High levels of polygyny in an insect pest population in which only a small number of males are sufficient to produce high numbers of offspring also contribute to failure of pheromone-based control strategies (Wang *et al.*, 2021).

However, failures in male annihilation and mating disruption have also in part been ascribed to the ability of *T. absoluta* to reproduce parthenogenetically, a phenomenon whereby females can produce embryos in the absence of males (Mittwoch, 1978). This form of reproduction was first documented in *T. absoluta* in a population collected in France in 2011 (Caparros Megido *et al.*, 2012). This species' ability to reproduce asexually was later also confirmed in Tunisian and Chinese populations of the pest (Abbes & Chermiti, 2014; Wang *et al.*, 2021).

5.2.2 Influence of *Wolbachia* on parthenogenesis in *Tuta absoluta*

The ability of *T. absoluta* to undergo deuterotokous parthenogenesis has long been speculated to be a consequence of manipulation by specific strains of the bacterial endosymbiont *Wolbachia* (Rickettsiales: Rickettsiaceae) (Abbes & Chermiti, 2014; Caparros Megido *et al.*, 2012). The maternally inherited endosymbiont *Wolbachia* has numerous strains affecting host reproductive biology in diverse ways, including, cytoplasmic incompatibility, parthenogenesis, male feminization and killing (Biondi *et al.*, 2018; Correa & Ballard, 2012; Engelstädter & Telschow, 2009). It is, however, the ability of *Wolbachia* to induce parthenogenetic reproduction that is of concern when management tools such as mating disruption and male annihilation are compromised.

South African *T. absoluta* populations screened for *Wolbachia* infection and the ability to undergo parthenogenesis (Chapter 3) exhibited similar findings to the study by Carvalho *et al.* (2018). The *Wolbachia* infection rate in South African *T. absoluta* populations was high (≥ 95%), and all populations were infected with a single *Wolbachia* strain (ST41). No parthenogenesis was found in *T. absoluta* from South Africa. The inadequate control reported with pheromone-based control strategies can therefore not be attributed to *Wolbachia*-induced parthenogenesis alone. The high incidence of a single strain *Wolbachia* infection is more commonly associated with cytoplasmic incompatibility, which provides future prospects of using this endosymbiont as a management tool in incompatibility programs.

The research of (Wang *et al.*, 2021) on polygyny in *T. absoluta* indicated that even a small number of males in a cropping system are sufficient to produce high numbers of offspring that are still able to inflict substantial damage to the tomato crop. Considering the low potential of parthenogenesis to produce viable offspring, control failure when using only pheromone-based strategies is therefore more likely the result of polygyny than parthenogenesis (Wang *et al.*, 2021). Since no parthenogenesis was observed in South African *T. absoluta* populations (Chapter 3), it can be assumed that polygyny most likely plays a large role in the failure of pheromone-based strategies in South Africa.

In all accounts of parthenogenesis in *T. absoluta*, none of the research aimed to determine the *Wolbachia* infection rate in *T. absoluta* populations or to identify the strains of *Wolbachia* associated with the *T. absoluta* populations in question (Abbes & Chermiti, 2014; Caparros Megido *et al.*, 2012; Wang *et al.*, 2021). Out of the populations that exhibited parthenogenesis, the French population had the highest hatch rate (39.9%) for unfertilized eggs, followed by the populations from Tunisia (11.36 – 17.02%) and China (1.1%) (Abbes & Chermiti, 2014; Caparros Megido *et al.*, 2012; Wang *et al.*, 2021). Isolated virgin females from the Tunisian and French populations were able to produce both male and female offspring (deuterotokous) with similar sex ratios reported in both studies (Abbes & Chermiti, 2014; Caparros Megido *et al.*, 2012). All previous research that confirmed parthenogenesis in *T. absoluta* reported similar observations, *viz*. that isolated virgin females produced fewer eggs, lower hatch rates and prolonged survival compared to mated females (Abbes & Chermiti, 2014; Caparros Megido *et al.*, 2012; Wang *et al.*, 2021).

High rates of *Wolbachia* infection (≥95%) were reported in *T. absoluta* populations from both Montenegro and Croatia in eastern Europe (Škaljac *et al.*, 2012). However, neither the *Wolbachia* strains associated with these populations were identified, nor was the ability to reproduce parthenogenetically, evaluated (Škaljac *et al.*, 2012). In Brazil, Carvalho *et al.* (2018) reported similarly high *Wolbachia* infection rates (70 – 100%), with a single *Wolbachia* strain (ST41) being predominant. However, none of the females were able to lay viable eggs (Carvalho *et al.*, 2018) and no link between *Wolbachia* infection and parthenogenesis was found.

5.2.3 *Wolbachia* as a management tool: Incompatible insect technique

The incompatible insect technique (IIT) is an autocidal strategy that relies on the principle of reducing female fertility by utilizing *Wolbachia* to induce cytoplasmic incompatibility (Baton *et al.*, 2021; Bourtzis *et al.*, 2014).

Cytoplasmic incompatibility (CI) is a form of conditional sterility whereby the fertilized eggs of females not infected with the same *Wolbachia* strain as the males with which they mated, will undergo embryonic death (Hurst & Frost, 2015; Sinkins, 2004; Werren *et al.*, 2008). However, if mated with males infected with the same

Wolbachia strain or with uninfected males, infected females will produce offspring naturally (Baton *et al.*, 2021). This will eventually lead to the extinction of uninfected females, potentially resulting in the complete replacement of the original uninfected host with *Wolbachia* infected individuals (Caspari & Watson, 1959; Fine, 1978) as was reported for the South African (Chapter 3) and Brazilian *T. absoluta* populations (Carvalho *et al.*, 2018). It is this naturally-occurring mechanism of host population suppression that is exploited by IIT (Baton *et al.*, 2021).

The incompatible insect technique holds promise for control of *T. absoluta*. The development and implementation of IIT is most advanced for mosquito control. However, IIT has been under consideration for the control of *Bactrocera oleae* (Rossi) (Diptera: Tephritidae) (Apostolaki *et al.*, 2011), *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) (Cattel *et al.*, 2018; Nikolouli *et al.*, 2018), *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) (Zabalou *et al.*, 2009) and *Glossina* spp. (Diptera: Glossinidae) (Bourtzis *et al.*, 2016).

The mass rearing and release of *T. absoluta* moths infected with a *Wolbachia* strain incompatible with the ST41 strain that dominates in the South African *T. absoluta* populations (Chapter 3), may also hold promise for management of this pest in South Africa. Mass-releases of incompatible males as part of an area-wide strategy could provide farmers with another environmentally friendly management tool.

5.3 Spinosad and insect resistance

With two modes of action, *viz*. depolarizing nicotinic acetylcholine and γ-aminobutyric acid (GABA) receptor neurons, spinosad demonstrates rapid contact and ingestion activity in insects which is unusual for a biological product (Kirst, 2010; Sparks *et al.*, 2012; Thompson *et al.*, 2000). It was originally believed that spinosyns with their unique mode of action, lack of cross-resistance, selectivity with regards to non-target insects, and moderate residual profile would greatly reduce the probability of resistance evolution, especially when applied in farming systems with effective insecticide rotation programs (Thompson *et al.*, 2000). However, the adaptability of insects has repeatedly been illustrated by the increasing numbers of reported cases of insecticide resistance (Sparks *et al.*, 2021; Thompson *et al.*, 2000). Although resistance management plans and resistance monitoring were put in place before the introduction of spinosad into the market (Salgado & Sparks, 2005; Thompson *et al.*, 2000; Zhao *et al.*, 2002), the indiscriminate use and overreliance resulted in the evolution of resistance. Field evolved spinosad resistance in *T. absoluta* was reported in Brazil, Chile and the United Kingdom (UK) (Campos *et al.*, 2014; Grant *et al.*, 2019; Reyes *et al.*, 2012). No reports of *T. absoluta* resistance to spinosad have yet been made in South Africa.

The South African *T. absoluta* population screened for susceptibility to spinosad by means of leaf dip assays (Chapter 4) had an estimated LC_{50} value of 0.41, and could therefore be considered as highly susceptible. The estimated LC_{50} values for second-instar larvae of four of *T. absoluta* populations screened for spinosad susceptibility in the United Kingdom, ranged between 149 ppm to more than 2500 ppm, considerably higher than that of a susceptible population screened from Spain (5.2 ppm) (Grant *et al.*, 2019). However, a population from Brazil, which was used in resistance development studies already showed signs of resistance in the field prior to the spinosad selection process, had a similar LC_{50} value (0.41 ppm) to that of the South African population (Campos *et al.*, 2014). Alarmingly, when the population from Brazil was selected for spinosad resistance it was able to evolve rapidly, reaching resistance levels of 180 000-fold after only seven generations of selection pressure (Campos *et al.*, 2014).

Spinosad remains one of the most promising insecticides for management of *T. absoluta*. Although it is only registered to be administered as a foliar application, limited root uptake and translaminar activity of spinosad has also been reported (Sparks *et al.*, 1999). An opportunity therefore exists to use this product as a systemic insecticide, provided that its systemic presence in plants does not have any adverse effects that would result in it not being registered as a systemic treatment for this pest.

5.3.1 Spinosad used as a systemic insecticide

The systemic activity of spinosad was discovered when it was added as a drench to tomato planted in rockwool (Van Leeuwen *et al.*, 2005). Plants with systemic spinosad toxicity were able to effectively control *Tetranychus urticae* (Koch) (Acari: Tetranychidae), *T. vaporariorum* and *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) (Van Leeuwen *et al.*, 2005; Van Leeuwen *et al.*, 2006). In this study (Chapter 4), similarly high toxicity was observed when second-instar *T. absoluta* larvae fed on tomato plants treated with systemically administered spinosad, with an estimated LC_{50} value of 9.35 ppm (1.87 mg a.i./plant) and an LC_{80} value of 20.1 ppm (4.02 mg a.i./plant).

The recommended rates at which spinosad is registered to be administered as a foliar application in South Africa range between 72 – 180 g/ha. With a plant density of 20 000 plants/ha in greenhouses it is estimated that the currently registered rates relate to 3.6 – 9 mg a.i./plant. Therefore, the registered rate of 9 mg a.i./plant will effectively control *T. absoluta* second-instar larvae for at least 25 DAT (Chapter 4). This persistence of systemic toxicity of spinosad was also reported by Van Leeuwen et al. (2005) against *T. urticae* after drenching tomato plants grown in rockwool with 4.8 mg a.i./plant. Control percentages of *T. urticae* decreased steadily over time, with a significant reduction in control only recorded 25 days after treatment (DAT) (Van Leeuwen *et al.*, 2005). Similarly, in this study (Chapter 4), plants drenched with 9.6 mg a.i./plant resulted in high mortality rates of *T. absoluta* larvae (≥96.51%) for at least 25 DAT, even though the spinosad concentrations within plants gradually decreased over time (Chapter 4).

This longer residual activity of spinosad when drenched into the root zone of tomato plants is in clear contrast with foliar applications, where spinosyn A and D rapidly dissipate with a half-life of 1.6 – 16 days (Saunders & Bret, 1997). The short persistence of spinosad in the crop environment and its selectivity with regards to non-target species were two of the many qualities that contributed to the appeal of spinosad for use in organic farming (Salgado *et al.*, 1998). Repeated application of a single insecticidal mode of action (Helps *et al.*, 2020) or application of persistent insecticides, as in the case of systemically applied spinosad, would increase the selection pressure for resistance due to increased exposure time (Van Leeuwen *et al.*, 2005; Van Leeuwen *et al.*, 2006).

The gradual decrease of spinosad concentration in plant tissue, as observed in this study (Chapter 4), and subsequent sub-lethal exposure provide selection pressure for the survival and reproduction of insects with resistant alleles (Van Leeuwen *et al.*, 2005). Sub-lethal exposure of *T. absoluta* larvae to spinosad might delay the selection for major single gene resistance (e.g. altered target site sensitivity), but will more likely favour multifactorial or polygenic resistance (e.g. behavioural avoidance, increased detoxification enzyme activity, etc.) leading to small increases in the magnitude of insecticide resistance (Gressel, 2011).

Larval instar-dependant tolerance was found in *T. absoluta* larvae from South Africa (Chapter 4), with the estimated LC_{80} values for later larval instars indicating a decrease in susceptibility to spinosad. The larvae used to assess the efficacy of plants with systemically acquired spinosad toxicity were second-instar larvae (Chapter 4), which had an estimated LC_{80} value of 0.97 ppm in the leaf dip bioassay. The estimated LC_{80} value for fourth-instar larvae was however 2.6-fold higher than second-instar larvae. Considering *T. absoluta* generational overlapping and the constant decrease in spinosad concentration within the plant tissue over time, it is highly likely that larvae (especially later instars) could at some stage be exposed to sub-lethal spinosad dosages when this insecticide is applied systemically.

It is, however, not only *T. absoluta* that will be influenced, but the entire arthropod species complex exposed to tomato with systemic toxicity. Therefore, resistance could also evolve in other tomato pests exposed to sub-lethal concentrations of spinosad for prolonged periods. Non-target effects of spinosad on beneficial insects such as predators and parasitoids of *T. absoluta* could however also be affected by feeding on prey or hosts that consumed plant material contaminated with spinosad (secondary poisoning) (Sanchez-Bayo *et al.*, 2013). In view of the above, the length of predator and parasitoid exposure to systemic insecticides plays a larger role than the concentration applied (Sanchez-Bayo *et al.*, 2013). It is important to remember that systemic insecticides contaminate all plant tissues including the pollen and nectar of flowers and could therefore also negatively impact pollinators (Lue *et al.*, 1984; Meher *et al.*, 2010; Sanchez-Bayo *et al.*, 2013).

Tomato plants drenched with spinosad at the seedling stage produced fruit with spinosad concentrations (80 DAT: <0.003 µg/g) (Chapter 4), well below the maximum residual limits mandated in South Africa (0.2 µg/g) and the European Union (0.7 µg/g). However, drenching spinosad at a later plant growth stage closer to harvest, could expose consumers to residues at higher concentrations. It is clear that although applying spinosad as a drench to induce systemic toxicity has many benefits, it also poses several challenges with regards to insect resistance management, non-target effects and possible consumer residual exposure.

5.4 Conclusions

The application of *B. bassiana* and *M. anisopliae* conidia as a soil drench resulted in high mortality of *T. absoluta* pupae. Additionally, EPF application as a soil drench resulted in a significant reduction in the fecundity of females that survived the EPF infection as pupae. This provides evidence of the suppressive potential when administering of EPF conidia applied as a soil drench for the control of the previously untargeted pupal stage of *T. absoluta*.

This study showed that all South African *T. absoluta* populations had high *Wolbachia* infection rates, nonetheless no parthenogenesis was observed. Control failure of pheromone-based strategies in South Africa can therefore not be attributed to *Wolbachia*-induced parthenogenesis, but is most likely caused by the high levels of polygyny in *T. absoluta*. The high frequency of a single strain *Wolbachia* throughout South African populations is more commonly associated with cytoplasmic incompatibility, which provides prospects of exploiting *Wolbachia* in incompatibility programmes, which warrant investigation in future studies.

The systemic application of spinosad, when drenched to the roots of tomato plants at concentrations similar to registered foliar application rates, resulted in the effective control of *T. absoluta* for an entire life cycle of the pest. When drenched at the seedling stage, tomato fruit contained spinosad at concentrations far lower than the mandated limits in South Africa. However, due to the persistent presence of spinosad in tomato plants, future studies should investigate the effects thereof on resistance evolution of the entire exposed pest complex and the impact on non-target organisms and subterranean and diversity.

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APPENDIX A

Instructions to authors: Insects

General Considerations

Research manuscripts should comprise:

- Front matter: Title, Author list, Affiliations, Abstract, Keywords
- Research manuscript sections: Introduction, Materials and Methods, Results, Discussion, Conclusions (optional).
- Back matter: Supplementary Materials, Acknowledgments, Author Contributions, Conflicts of Interest, References.

Review manuscripts should comprise the front matter, literature review sections and the back matter. The template file can also be used to prepare the front and back matter of your review manuscript. It is not necessary to follow the remaining structure. Structured reviews and meta-analyses should use the same structure as research articles and ensure they conform to the PRISMA guidelines.

Graphical Abstract:

A graphical abstract (GA) is an image that appears alongside the text abstract in the Table of Contents. In addition to summarizing the content, it should represent the topic of the article in an attention-grabbing way. Moreover, it should not be exactly the same as the Figure in the paper or just a simple superposition of several subfigures. Note that the GA must be original and unpublished artwork. Any postage stamps, currency from any country, or trademarked items should not be included in it.

The GA should be a high-quality illustration or diagram in any of the following formats: PNG, JPEG, TIFF, or SVG. Written text in a GA should be clear and easy to read, using one of the following fonts: Times, Arial, Courier, Helvetica, Ubuntu or Calibri.

The minimum required size for the GA is 560 × 1100 pixels (height × width). The size should be of high quality in order to reproduce well.

- Acronyms/Abbreviations/Initialisms should be defined the first time they appear in each of three sections: the abstract; the main text; the first figure or table. When defined for the first time, the acronym/abbreviation/initialism should be added in parentheses after the written-out form.
- SI Units (International System of Units) should be used. Imperial, US customary and other units should be converted to SI units whenever possible.
- Accession numbers of RNA, DNA and protein sequences used in the manuscript should be provided in the Materials and Methods section. Also see the section on Deposition of Sequences and of Expression Data.
- Equations: If you are using Word, please use either the Microsoft Equation Editor or the MathType add-on. Equations should be editable by the editorial office and not appear in a picture format.
- Research Data and supplementary materials: Note that publication of your manuscript implies that you must make all materials, data, and protocols associated with the publication available to readers. Disclose at the submission stage any restrictions on the availability of materials or information. Read the information about Supplementary Materials and Data Deposit for additional guidelines.
- Preregistration: Where authors have preregistered studies or analysis plans, links to the preregistration must be provided in the manuscript.
- Guidelines and standards: MDPI follows standards and guidelines for certain types of research. See https://www.mdpi.com/editorial_process for further information.
- New Species Description: Manuscripts that describe new or revised taxon names must be registered in ZooBank, as required by the International Code of Zoological Nomenclature, after article acceptance following peer review. This ensures that your article is officially recorded as the first paper to describe the new species. The ZooBank unique identification code (LSID—Life Science Identifier) should be provided at the final proofreading stage, on the first page of your manuscript, following the affiliations, so that it is included in your published article. An LSID is represented as a uniform resource name (URN) with the following format:

Front Matter

These sections should appear in all manuscript types

- Title: The title of your manuscript should be concise, specific and relevant. It should identify if the study reports (human or animal) trial data, or is a systematic review, meta-analysis or replication study. When gene or protein names are included, the abbreviated name rather than full name should be used.
- Author List and Affiliations: Authors' full first and last names must be provided. The initials of any middle names can be added. The PubMed/MEDLINE standard format is used for affiliations: complete address information including city, zip code, state/province, and country. At least one author should be designated as corresponding author, and his or her email address and other details should be included at the end of the affiliation section. Please read the criteria to qualify for authorship.
- Simple Summary: It is vitally important that scientists are able to describe their work simply and concisely to the public, especially in an open-access on-line journal. The simple summary consists of no more than 200 words in one paragraph and contains a clear statement of the problem addressed, the aims and objectives, pertinent results, conclusions from the study and how they will be valuable to society. This should be written for a lay audience, i.e., no technical terms without explanations. No references are cited and no abbreviations. Submissions without a simple summary will be returned directly. Example could be found at https://www.mdpi.com/2075-4450/11/8/508.
- Abstract: The abstract should be a total of about 200 words maximum. The abstract should be a single paragraph and should follow the style of structured abstracts, but without headings: 1) Background: Place the question addressed in a broad context and highlight the purpose of the study; 2) Methods: Describe briefly the main methods or treatments applied. Include any relevant preregistration numbers, and species and strains of any animals used. 3) Results: Summarize the article's main findings; and 4) Conclusion: Indicate the main conclusions or interpretations. The abstract should be an objective representation of the article: it must not contain results which are not

presented and substantiated in the main text and should not exaggerate the main conclusions.

- Keywords: Three to ten pertinent keywords need to be added after the abstract. We recommend that the keywords are specific to the article, yet reasonably common within the subject discipline.

Research Manuscript Sections

- Introduction: The introduction should briefly place the study in a broad context and highlight why it is important. It should define the purpose of the work and its significance, including specific hypotheses being tested. The current state of the research field should be reviewed carefully and key publications cited. Please highlight controversial and diverging hypotheses when necessary. Finally, briefly mention the main aim of the work and highlight the main conclusions. Keep the introduction comprehensible to scientists working outside the topic of the paper.
- Materials and Methods: They should be described with sufficient detail to allow others to replicate and build on published results. New methods and protocols should be described in detail while well-established methods can be briefly described and appropriately cited. Give the name and version of any software used and make clear whether computer code used is available. Include any pre-registration codes.
- Results: Provide a concise and precise description of the experimental results, their interpretation as well as the experimental conclusions that can be drawn.
- Discussion: Authors should discuss the results and how they can be interpreted in perspective of previous studies and of the working hypotheses. The findings and their implications should be discussed in the broadest context possible and limitations of the work highlighted. Future research directions may also be mentioned. This section may be combined with Results.
- Conclusions: This section is mandatory, and should provide readers with a brief summary of the main achievements/results of your work.
- Patents: This section is not mandatory, but may be added if there are patents resulting from the work reported in this manuscript.

Back Matter

- Supplementary Materials: Describe any supplementary material published online alongside the manuscript (figure, tables, video, spreadsheets, etc.). Please indicate the name and title of each element as follows Figure S1: title, Table S1: title, etc.
- Funding: All sources of funding of the study should be disclosed. Clearly indicate grants that you have received in support of your research work and if you received funds to cover publication costs. Note that some funders will not refund article processing charges (APC) if the funder and grant number are not clearly and correctly identified in the paper. Funding information can be entered separately into the submission system by the authors during submission of their manuscript. Such funding information, if available, will be deposited to FundRef if the manuscript is finally published. Please add: “This research received no external funding” or “This research was funded by [name of funder] grant number [xxx]” and “The APC was funded by [XXX]” in this section. Check carefully that the details given are accurate and use the standard spelling of funding agency names at https://search.crossref.org/funding, any errors may affect your future funding.
- Acknowledgments: In this section you can acknowledge any support given which is not covered by the author contribution or funding sections. This may include administrative and technical support, or donations in kind (e.g., materials used for experiments).
- Author Contributions: Each author is expected to have made substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data; or the creation of new software used in the work; or have drafted the work or substantively revised it; AND has approved the submitted version (and version substantially edited by journal staff that involves the author’s contribution to the study); AND agrees to be personally accountable for the author’s own contributions and for ensuring that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and documented in the literature. For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, X.X.

and Y.Y.; Methodology, X.X.; Software, X.X.; Validation, X.X., Y.Y. and Z.Z.; Formal Analysis, X.X.; Investigation, X.X.; Resources, X.X.; Data Curation, X.X.; Writing – Original Draft Preparation, X.X.; Writing – Review & Editing, X.X.; Visualization, X.X.; Supervision, X.X.; Project Administration, X.X.; Funding Acquisition, Y.Y.”, please turn to the CRediT taxonomy for the term explanation. For more background on CRediT, see here. "Authorship must include and be limited to those who have contributed substantially to the work. Please read the section concerning the criteria to qualify for authorship carefully".

- Institutional Review Board Statement: In this section, please add the Institutional Review Board Statement and approval number for studies involving humans or animals. Please note that the Editorial Office might ask you for further information. Please add “The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of NAME OF INSTITUTE (protocol code XXX and date of approval).” OR “Ethical review and approval were waived for this study, due to REASON (please provide a detailed justification).” OR “Not applicable” for studies not involving humans or animals. You might also choose to exclude this statement if the study did not involve humans or animals.
- Data Availability Statement: In this section, please provide details regarding where data supporting reported results can be found, including links to publicly archived datasets analyzed or generated during the study. Please refer to suggested Data Availability Statements in section “MDPI Research Data Policies”. You might choose to exclude this statement if the study did not report any data.
- Conflicts of Interest: Authors must identify and declare any personal circumstances or interest that may be perceived as influencing the representation or interpretation of reported research results. If there is no conflict of interest, please state "The authors declare no conflict of interest." Any role of the funding sponsors in the choice of research project; design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript; or in the decision to publish the results must be declared in this section. *Insects* does not publish studies funded partially or fully by the

tobacco industry. Any projects funded by industry must pay special attention to the full declaration of funder involvement. If there is no role, please state "The sponsors had no role in the design, execution, interpretation, or writing of the study". For more details please see Conflict of Interest.

- References: References must be numbered in order of appearance in the text (including table captions and figure legends) and listed individually at the end of the manuscript. We recommend preparing the references with a bibliography software package, such as EndNote, Reference Manager or Zotero to avoid typing mistakes and duplicated references. We encourage citations to data, computer code and other citable research material. If available online, you may use reference style 9. below.
- Citations and References in Supplementary files are permitted provided that they also appear in the main text and in the reference list.

In the text, reference numbers should be placed in square brackets [ ], and placed before the punctuation; for example [1], [1–3] or [1,3]. For embedded citations in the text with pagination, use both parentheses and brackets to indicate the reference number and page numbers; for example [5] (p. 10). or [6] (pp. 101–105).

The reference list should include the full title, as recommended by the ACS style guide. Style files for Endnote and Zotero are available.

References should be described as follows, depending on the type of work:

1. Journal Articles: Author 1, A.B.; Author 2, C.D. Title of the article. Abbreviated Journal Name Year, Volume, page range.

2. Books and Book Chapters: Author 1, A.; Author 2, B. Book Title, 3rd ed.; Publisher: Publisher Location, Country, Year; pp. 154–196. Author 1, A.; Author 2, B. Title of the chapter. In Book Title, 2nd ed.; Editor 1, A., Editor 2, B., Eds.; Publisher: Publisher Location, Country, Year; Volume 3, pp. 154–196.

3. Unpublished work, submitted work, personal communication: Author 1, A.B.; Author 2, C. Title of Unpublished Work. status (unpublished; manuscript in preparation). Author 1, A.B.; Author 2, C. Title of Unpublished Work. Abbreviated Journal Name stage of publication (under review; accepted; in press). Author 1, A.B. (University, City, State, Country); Author 2, C. (Institute, City, State, Country). Personal communication, Year.

4. Conference Proceedings: Author 1, A.B.; Author 2, C.D.; Author 3, E.F. Title of Presentation. In Title of the Collected Work (if available), Proceedings of the Name of the Conference, Location of Conference, Country, Date of Conference; Editor 1, Editor 2, Eds. (if available); Publisher: City, Country, Year (if available); Abstract Number (optional), Pagination (optional).

5. Thesis: Author 1, A.B. Title of Thesis. Level of Thesis, Degree-Granting University, Location of University, Date of Completion.

6. Websites: Title of Site. Available online: URL (accessed on Day Month Year). Unlike published works, websites may change over time or disappear, so we encourage you create an archive of the cited website using a service such as WebCite. Archived websites should be cited using the link provided as follows: Title of Site. URL (archived on Day Month Year).

Preparing Figures, Schemes and Tables

- File for Figures and Schemes must be provided during submission in a single zip archive and at a sufficiently high resolution (minimum 1000 pixels width/height, or a resolution of 300 dpi or higher). Common formats are accepted, however, TIFF, JPEG, EPS and PDF are preferred.
- *Insects* can publish multimedia files in articles or as supplementary materials. Please contact the editorial office for further information.
- All Figures, Schemes and Tables should be inserted into the main text close to their first citation and must be numbered following their number of appearance (Figure 1, Scheme I, Figure 2, Scheme II, Table 1, *etc.*).
- All Figures, Schemes and Tables should have a short explanatory title and caption.
- All table columns should have an explanatory heading. To facilitate the copy-editing of larger tables, smaller fonts may be used, but no less than 8 pt. in size. Authors should use the Table option of Microsoft Word to create tables.
- Authors are encouraged to prepare figures and schemes in color (RGB at 8-bit per channel). There is no additional cost for publishing full color graphics.

APPENDIX B

Instructions to authors: *Entomologia Generalis*

Aims and Scope (see more details in Desneux & Biondi, 2018, *Entomologia Generalis* 37(1), 1–5).

Entomologia Generalis welcomes high-quality contributions from the field of basic and applied ecology of arthropods, insects and mite pests, as well as their natural enemies and pollinators. Articles published in *Entomologia Generalis* should not be descriptive, but should bring novel findings on topics of current importance.

Article types:

- Original Research Articles Such articles are high quality research articles on advances in knowledge on fields covered by the journal (see scope). They should bring valuable and original insights in key research areas and they are expected to have a broad and rapid impact on the scientific community working in the fields of entomology and ecology of arthropods.
- Review Articles They should provide significant developments in the field of entomology and ecology of arthropods. Although review papers are usually solicited by members of the editorial board, non-solicited review articles may be considered for publication in *Entomologia Generalis*. Please send proposals to the Editor-in-Chief (Dr. Nicolas Desneux, nicolas.desneux@univ-cotedazur.fr) for preliminary assessment by the editorial board team before formal submission to the journal.
- Comments The journal also publishes comments and rebuttals on papers previously published in *Entomologia Generalis*. Such documents should first be discussed with the editors and are not to be submitted directly to the journal.

Article length: Word counts given below include abstract (250 words maximum), main text, acknowledgments, references (30 references accounting for 900 words), tables and figures (half page table or figure accounting for 450 words and full-page figure or table accounting for 900 words).

- Research articles: maximum 7000 words.
- Review articles: maximum 13000 words.
- Letters and Short notes: maximum 3000 words.
- Comments: maximum 2500 words.

Manuscript Formatting:

- Manuscripts should be submitted in MS word format i.e. .doc or.docx.
- Acceptable files: main document: .doc and .docx; tables: editable tables at the end of the main document as doc and .docx; figures: jpg, tif, pdf or any vectorized file format.
- Page and line numbering (MS Word numbering option) must be used continuously throughout the entire manuscript.
- Please use double-line spacing for the text.
- 12-point Times Roman should be used throughout the whole manuscript.
- Page format should be set up at 2.5 cm margins in left and right sides as well as top and bottom of the page.
- The text of the entire manuscript must be left justified.
- Use tab stops or other commands for indents, not spaces.
- Up to three levels of headings are accepted.
- Use the MS equation editor or MathType for equations.
- Figure legends must be numbered and provided at the end of the main document. Make sure to cite each table and figure at least once in the main text using the wording Fig. and Table.
- Tables should be prepared using the table function in MS Word, do not use spread sheets.
- Genus and species names must be written in italics. Spell out the genus name only at the first mentioning in the main text and also when appearing at the beginning of a sentence throughout the manuscript.
- Abbreviations can be used pending they are defined at first time they appear, abbreviations should be used consistently throughout the manuscripts.
- Sentences with personal reference to the authors (I, We, Our) should be avoided, passive voice is preferred.
- Footnotes are not allowed in main text, still they can be used in Tables.

Language:

- All contributions should be written in either American or British English language. The English type should be consistent throughout the entire manuscript.
- Authors for whom English is a second language should have their manuscript edited before submission, either by a professional service or with the help of a native English speaker. Manuscripts with poor English not understandable for reviewers will be returned to authors without review. Accepted manuscripts may still have to be polished for English before final publication; Schweizerbart Science Publishers can provide such service at reasonable costs.

Manuscript structure:

Title page

- Title: must be concise and informative. For organism names, please give the common or latin name but no authority or order and family. Do not use capital letters for the first letter or each word of the title.
- A short title (max. 45 characters) must be added below the full title.
- Author(s) name(s): Include first the given name, the initial(s) of the middle name(s) then the family name. Check that all names are accurately spelled.
- Authors shall provide their affiliation(s) and full address(es), a valid e-mail address and telephone number(s).

Abstract

- Provide an abstract of up to 250 words. The abstract should be self-explaining and should summarize the conceptual framework and aim of the work, the main results and conclusions. Moreover, it should not contain any undefined abbreviations or unspecified references. All Latin names should be provided with the correct authority and if applicable with (Order: Family).

Keywords

- Provide a minimum of 5 keywords which can be used for indexing purposes. Avoid words already present in the title and include the family of the most important organism(s) in the paper (e.g., those referred to in the title).

Main body for Research Articles (starting page 3)

- Divide your manuscript into clearly divided sections following strictly the order: Introduction, Material and methods, Results, Discussion, Acknowledgments, References, Figure legends, Tables (one per page), Figures (one figure per page or one figure panel per page). Sections should not be numbered.
- Each heading should appear on its own separate line. Subsections (subheadings) can be used only in Material and methods and Results sections. Subsections should have a brief title and no more than two sublevels are allowed.
- Introduction: Provide an adequate background, formulate the hypothesis(es), state the objectives of the work. Avoid a detailed literature survey or a summary of the results.
- Material and methods: Provide clear details to enable the work to be reproduced or expanded. Methods already published should be indicated by reference(s), and mostly relevant modifications should be described.
- Results: Be clear and concise. Do not repeat and/or list all the data presented in figures and/or in tables, but mention and describe the significant and striking ones. Ideally, the results should have subsections matching those of the Material and methods section.
- Discussion: The text should explore and discuss thoroughly the significance of the results. Do not repeat the results and do not list studies from other authors without deep integrative text legitimating the citations. Do not over-speculate on results. Combining Results and Discussion sections is not accepted in *Entomologia Generalis.* Ideally, the main conclusions of the study should be presented in a short paragraph at the end of the Discussion section.
- Acknowledgements should be given as a brief statement following the Discussion section. They may refer to any technical, scientific or linguistic help received for the work by colleagues, and/or by professionals and/or by the editor(s) and anonymous referees. Authors should use this section to acknowledge any funding, citing the funding source, the project title/acronym and grant number (if any).
- Citation in the text. Cite references in the text by family name of the first author and year in parentheses, and use the format provided here as template. Examples: “Several studies supported this hypothesis (Abbott 1991;

> Smith et al. 2001; 2002a; 2002b; Thompson et al. 2010). The samples were analyzed using the procedures by Heimpel et al. (2012) and modified by Peterson (2015). Plants belonging to the *Rubus* genus are considered the main hosts for this insect (Wang & Lee 2016).

Reference list. The list of references should only include works that are cited in the text and that have been published, in press or accepted for publication. Personal communications and unpublished works are generally not accepted in the text and thus should not be given as references. Reference list entries should be alphabetized by the last names of the first author of each work. List multi-author publications of the same first author in chronological order. When authors cite multiple papers authored by the same first author and published in the same year, the year should be followed by a letter, using “a” for the first cited paper, “b” for the second and so on. Ideally, the names of all authors should be provided, the usage of “et al.” in long author lists (longer than six authors) can also be used. We strongly recommend authors to use the APA 6 reference style to format references. Authors not using EndNote shall follow the APA 6 reference formatting style. If possible, please also supply the Endnote reference file.

Journal Article:

Ito, L., Omori, T., Yoneda, M., Yamaguchi, T., Kobayashi, R., & Takahashi, Y. (2018). Origin and migration of trace elements in the surface sediments of Majuro Atoll, Marshall Islands. *Chemosphere*, 202, 65–75. https://doi.org/10.1016/j.chemosphere.2018.03.083

Book:

Aitken, C. G. G., & Taroni, F. (2004). *Statistics and the Evaluation of Evidence for Forensic Scientists*. J. Wiley & Sons Ltd. https://doi.org/10.1002/0470011238

Book Chapter:

Wantzen, K. M., Callil, C., & Butakka, C. M. M. (2011). Benthic invertebrates of the Pantanal and its tributaries. In W. J. Junk, C. J. Silva, C. N. Cunha, & K. M. Wantzen (Eds.), *The Pantanal: Ecology, biodiversity and sustainable management of a large neotropical seasonal wetland* (pp. 127–141). Sofia, Moscow: Pensoft.

Thesis:

Králík, M. (2004). Paleodermatoglyphics. *Analysis of fingerprintson ancient ceramics: theoretical basis, methodological issues and practical recommendations*. PhD thesis, Masaryk University in Brno.

Web:

Haq, B. U. (2018): Jurassic Sea-Level Variations: A Reappraisal. GSA Today. https://doi.org/10.1130/GSATG359A.1

Other:

Rivaldería, N., Expósito, N., Gómez-Herrero, F. J., Juanino, R. M., Cruz, R., Hernández- Hurtado, L. E., . . . Alonso-Rodríguez, C. G. R. (2017). Diferencias intra e interpoblacionales en la frecuencia de apar-ición de los distintos tipos de deltas (o trirradii) dactilares. *XX Congreso Sociedad Española de Antropología Física*.

APPENDIX C

Instructions to authors: *Journal of Economic Entomology*

Formatting

For new submissions, our formatting requirements are simple—just make sure your paper has the following items:

- Continuous line numbers.
- Double-spaced lines.
- A title page and abstract in the main document.
- A main document in a doc, docx, tex (converted to PDF for review), or rtf file type.
- Tables in a Word document (we cannot accept Excel files, unless they are supplementary files).
- Figure and table legends in the main document.
- All co-authors entered into the online review system (email addresses required).

Please note there are more formatting guidelines for revised versions, as those are closer to being accepted (see the Revised Versions section of these author instructions).

- References listed in alphabetical order, cited by author and year in the text (not numbered)
- Figures and tables at the end of the main document after the references, or uploaded as separate files. Figure legends should be included at the end of the main text after the references, and table legends should be next to their corresponding tables
- Text is single-column

Article types and lengths

- Research article: No limit (under 7500 words recommended)
- Review: No limit
- Forum: No limit

- Short Communication: 2,000 words or less (not including abstract, references, or figure and table legends)
- Letter to the Editor: 2,000 words or less

For explanations of the different article types, please see the Article Types section of these author instructions.

Language

- English. A second abstract in a second language is permitted. Authors are responsible for the accuracy of non-English abstracts.
- Manuscripts with poor English will be withdrawn before review. Those authors are encouraged to pursue English assistance prior to resubmission. For a professional editing service, ESA journals have a partnership with the Charlesworth Group, whereby ESA members receive a 10% discount on editing services. The discount is automatically applied if authors follow the hyperlink above.

Body

Introduction

Clearly state the basis of your study along with background information and a statement of purpose.

Materials and Methods

Include a clear and concise description of the study design, experiment, materials, and method of statistical analysis.

Results

Clearly present the results. Do not include interpretation of results or interpretation of statistical analysis—simply present the results of the experiment and the results of the statistical analysis. Data listed in tables should not be listed in the results; instead, refer to the table.

Discussion

Interpret and discuss results of the study and their implications. Include suggestions for direction of future studies, if appropriate.

Acknowledgments

Place the acknowledgments after the text. Organize acknowledgments in paragraph form in the following order: persons, groups, granting institutions, grant numbers, and serial publication number. Following the Acknowledgments, you may include a statement of author contribution outlining the specific contributions of each author to the article. A statement of author contribution is welcomed but not required.

References

- EndNote style is “Environmental Entomology,” and Reference Manager style is “Journal of Medical Entomology.”
- Only cite published or formally accepted (in press) articles, not submitted articles.
- References should be in alphabetical order. If multiple references from the same author are cited, those references should be in chronological order.
- Abbreviate journal titles according to the most recent issue of BIOSIS Serial Sources.
- For non-English titled journals that are cited in the references, the title of the journal should be spelled out.
- Systematics-related articles may specify that all serial titles be spelled out for final publication.

Sample reference styles

Journal Articles

Evans, M. A. 2000. Article title: subtitle (begin with lowercase after colon or dash unless first word is a proper noun). J. Abbr. 00:000–000.

Evans, M. A. 2001a. Article title. J. Abbr. 00: 000–000.

Evans, M. A., and R. Burns. 2001. Article title. J. Abbr. 00: 000–000.

Evans, M. A., and A. Tyler. 2001. Article title. J. Abbr. 00: 000–000.

Evans, M. A., A. Tyler, and H. H. Munro. 2000. Article title. J. Abbr. 00: 000–000.

Evans, M. A., R. Burns, and A. A. Dunn. 2001. Article title. J. Abbr. 00: 000–000.

In Press

Evans, M. A. 2002. Article title. J. Econ. Entomol. (in press).

Books

Burns, R. 2001. Title (initial cap only): subtitle (no initial cap after colon). Publisher, city, state abbreviation or country.

Evans, M. A. 2001. Colorado potato beetle, 2nd ed. Publisher, city, state abbreviation or country.

Article/Chapter in Book

Tyler, A. 2001. Article or chapter title, pp. 000–000. In T.A.J. Royer and R. B. Burns (eds.), Book title. Publisher, city, state abbreviation or country.

Tyler, A., R.S.T. Smith, and H. Brown. 2001. Onion thrips control, pp. 178–195. In R. S. Green and P. W. White (eds.), Book title, vol. 13. Entomological Society of America, Lanham, MD.

No Author Given

(USDA) U.S. Department of Agriculture. 2001. Title. USDA, Beltsville, MD.

(IRRI) International Rice Research Institute. 2001. Title. IRRI, City, State or Country.

Patents

Harred, J. F., A. R. Knight, and J. S. McIntyre, inventors; Dow Chemical Company, assignee. 1972 Apr 4. Epoxidation process. U.S. patent 3,654,317.

Theses/Dissertations

James, H. 2001. Thesis or dissertation title. M.S. thesis or Ph.D. dissertation, University of Pennsylvania, Philadelphia.

Software

SAS Institute. 2001. PROC user's manual, version 6th ed. SAS Institute, Cary, NC.

Online Citations

Reisen, W. 2001. Title. Complete URL (protocol://host.name/path/file.name) and/or DOI (Digital Object Identifier)

Appendix D

Declaration of language editing

Language editing statement

To whom this may concern,
I, Prof. J van den Berg, hereby declare that the thesis titled: “The potential of biopesticides for control of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae)” by R. Erasmus, has been checked by the supervisors for language correctness and spelling. The literature references are considered the responsibility of the student.

J van den Berg

Prof. J van den Berg
3 December 2021