EVALUATION OF ENTOMOPATHOGENS AND PLANT EXTRACTS AS OPTIONS FOR INTEGRATED PEST MANAGEMENT OF *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae) FOR ENHANCED TOMATO PRODUCTIVITY IN RWANDA

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DECLARATION AND RECOMMENDATION

Declaration

This thesis is my original work and has not been presented in this University or any other for the award of a degree.

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DEDICATION

This work is dedicated to my loving and caring wife, Pauline Mutuyimana, and our children: Reagan Mugisha, Ketia Ganza, and Ian Nziza, who have been and will always be my comforters and the source of my strength.

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ABSTRACT

Tomato (*Solanum lycopersicum* L.) is an economically and nutritionally important crop in Rwanda. However, its production is threatened by the invasive tomato leaf miner (*Tuta absoluta* Meyrick) since 2015. Options for integrated pest management (IPM) for its control under Rwandan conditions have not been developed. The main objective of this study was to contribute to enhanced tomato productivity and fruit quality in Rwanda through evaluation of entomopathogens and plant extracts as options for integrated management of *T. absoluta.* Bioassay experiments were conducted at the Rwanda Agriculture and Animal Resources Development Board to determine: the potential of entomopathogenic nematode (EPN) isolates from Rwanda to control *T. absoluta*, the pathogenicity of selected commercial formulations of entomopathogenic fungi (EPFs) and the bioactivity of local plant extracts (PEs) against the pest. The two most effective EPNs, EPFs, and PEs were further evaluated in two field trials to determine their efficacy against *T. absoluta* infestation and their effects on tomato growth, yield, and fruit quality. The results indicated that all evaluated local EPN isolates caused high *T. absoluta* larval mortality (53.3% - 96.7%) only 24 h after inoculation and the mortality reached 100% after three days. The outstanding isolates were *Steinernema* sp. RW14-M-C2a-3 and *Steinernema* sp. RW14-M-C2b-1. The evaluated EPFs were also pathogenic against *T. absoluta* with Metatech® WP (*Metarhizium anisopliae,* Strain FCM Ar 23B3) and Beauvitech® WP (*Beauveria bassiana,* Strain J25) recording the highest mortality rates (82.8% and 60.8%) and LT_{50} values of 3.9 and 5.2 days, respectively. The highest *T. absoluta* larval mortality rates recorded five days after treatment with plant extracts were 35.1% and 24.9% for *Tephrosia vogelii* and *Phytolacca dodecandra*, respectively while azadirachtin caused more than 64% after only two days and 100% after five days. Under field conditions, the entomopathogens (EPNs and EPFs) and azadirachtin exhibited higher efficacy than the plant extracts and the controls with the maximum leaflet damage obtained 10 weeks after transplanting varying between 59.7% and 74.7% while plots treated with the synthetic insecticide, imidacloprid (positive control) recorded 80.0% - 92.1% damage. The entomopathogens and azadirachtin also increased the yield of healthy fruits per plant (average of two trials) 4.8, 4.5, 4.2, 4.1 and 5.0 folds for *Steinernema* sp. RW14-M-C2a-3, *Steinernema* sp. RW14-M-C2a-3, Metatech® WP, Beauvitech WP, and azadirachtin, respectively, as compared to the positive control. These entomopathogens and azadirachtin are effective against the pest without compromising fruit quality and should be included in IPM of *T. absoluta* in Rwanda. Further studies are recommended on their possible combinations and efficacy under greenhouse conditions.

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LIST OF ABBREVIATIONS AND ACRONYMS

CFU : Colony Forming Unit

CRD : Completely Randomized Design

EPF : Entomopathogenic Fungus

EPN : Entomopathogenic Nematode

EPPO : European and Mediterranean Plant Protection Organization

FAO : Food and Agriculture Organization

HSD Honestly Significant Difference

IJs : Infective Juveniles

IPM : Integrated Pest Management

MINAGRI: Ministry of Agriculture and Animal Resources

RAB : Rwanda Agriculture and Animal Resources Development Board

RCBD : Randomized Complete Block Design

RHOS : Rwanda Horticulture Organisations Survey

USAID : United States Agency for International Development

CHAPTER ONE

INTRODUCTION

1.1 Background information

Vegetables form an integral part of a balanced diet that provides various nutritional and antioxidant compounds required for good human health (Havard, 2019). Insufficient consumption of vegetables was reported to be associated with several chronic diseases, accelerated ageing and early mortality (Forouzanfar *et al*., 2016). The daily vegetable intake recommended by the world health organisation is 250 g per person (Trichopoulou *et al*., 2001). Therefore, any effort targeting vegetable promotion is of great value.

Tomato (*Solanum lycopersicum* L.) is one of the most commonly cultivated vegetable species. It is listed among the most important protective food as the fruits are rich in calcium (48 mg/100 g), sodium (12.9 mg/100 g), copper (0.19 mg/100 g), trace elements, vitamin A (900 IU), vitamin C (27 mg/100 g), vitamin B complex, essential amino-acids, and healthy organic acids like citric, formic, and acetic acids. Tomatoes are good appetisers and prevent cancer owing to lycopene and carotene content (Gopalakrishnan, 2007).

According to FAO (2019), the world tomato production was 182,301,395 t from an area of 4,848,384 ha during the year 2017. The five top world tomato producers are China (59,514,773 t), India (20,708,000 t), Turkey (12,750,000 t), USA (10,910,990 t) and Egypt (7,297,108 t). In Africa, tomato production in the year 2017 was 21,486,541 t from 1,303,148 ha; whereas 1,998,098 t were produced in East Africa from 153,940 ha. In Rwanda, tomato is the principal horticultural crop, produced throughout the year by small and medium scale growers for home consumption and income generation (Clay and Turatsinze, 2014). Tomato production in Rwanda was 117,732 t from an area of 8,396 ha during the year 2014 compared to only 8,000 t from 1150 ha in 2000, which indicates that the crop gained importance in the country. However, in the year 2017, the production declined (97,426 t) despite the increase in area under production (11,329 ha) (FAO, 2019).

Tomato crop has been reported to be attacked by different pests and diseases that have the potential to drastically reduce its yield (Kumar and Omkar, 2018). An invasive tomato leaf miner, *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae), threatens tomato production in Rwanda since its first detection in the country in the year 2015 (FAO, 2015). This pest is indisputably one of the main reasons for the registered tomato yield decline in Rwanda.

Tuta absoluta has about 12 generations per year and 250 to 300 eggs can be laid by one female in her lifespan. It attacks all aerial parts of tomato plant and can result in tomato yield losses up to 100% in greenhouse and open-field (Desneux *et al*., 2010). Besides tomato, the host range of *T. absoluta* includes plants in: Solanaceae family (*Capsicum annuum* L., *Solanum melongena* L., *S. tuberosum* L., *Nicotiana tabacum* L., *Datura stramonium* L.), Fabaceae family (*Phaseolus vulgaris* L., *Medicago sativa* L.), Amaranthaceae family (*Chenopodium rubrum* L., *Spinacia oleracea* L.), and others (Ferracini *et al*., 2012; Mansour *et al*., 2018). The ability of *T. absoluta* to invade secondary hosts in absence of tomato, its high heat tolerance and overwintering capacities enable it to quickly multiply over time, which complicates its management (Garzia *et al*, 2012). Since *T. absoluta* is a major threat to tomato production in Rwanda, it is important to develop sustainable strategies for its management to sustain the role of tomato in diversifying the economy, alleviating poverty, and improving nutrition (Clay and Turatsinze, 2014).

Chemical control is the main method used to manage *T. absoluta* in Sub-Saharan Africa, including Rwanda, due to lack of alternative measures (Mansour *et al*., 2018). However, limitations of this method to control the pest in different parts of the world have been reported due to several reasons: the endophytic feeding habit of *T. absoluta* larvae (in leaf mines, stems, and fruits), ability to develop resistance to repeatedly used pesticides, and high reproductive potential resulting from short regeneration time among others (Garzia *et al*., 2012; Roditakis *et al*., 2015; Biondi *et al*., 2018; Mansour *et al*., 2018). Besides, the use of synthetic insecticides often leads to several adverse environmental effects (Macharia *et al*., 2009; Moshi and Matoju, 2017). Furthermore, following the establishment of a pest in a new locality, the existing natural enemies will need time to get adapted to it and they may finally help in its control as it happened in the Mediterranean region (Desneux *et al*., 2010). Synthetic pesticides were however reported to disturb this natural equilibrium (Urbaneja *et al*., 2012).

The development of alternative options for management of *T. absoluta* is thus, regarded as a worldwide priority (Biondi *et al*., 2018; Mansour *et al*., 2018). The biorational control agents such as entomopathogenic fungi (EPFs), entomopathogenic nematodes (EPNs) and plant extracts would provide sustainable management of *T. absoluta* due to reduced risks associated with their use (Jallow *et al*., 2019; Isman, 2020; Nishi *et al*., 2020). Reports also indicated that they are among the best candidates (Shalaby *et al*., 2013; Van Damme *et al*., 2016; Salama and Shehata, 2017).

Though biopesticides based on the EPFs *Beauveria bassiana* (Bals.) Vuill. (Ascomycota: Hypocreales) and *Metarhizium anisopliae* (Metsch.) Sorok (Ascomycota: Hypocreales) have been studied on *T. absoluta*, commercial formulations recommended against this pest are limited (Biondi *et al.*, 2018). According to the same authors, reports on their use against *T. absoluta* in commercial greenhouse and field conditions are scarce. Besides, four EPNs isolated in Rwanda and other two exotic EPN species, maintained in Rwanda (Yan *et al*., 2016), have not been evaluated against *T. absoluta.* Insecticides of plant origin like azadirachtin were also reported as effective against *T. absoluta* (Yalçin *et al*., 2015*)*, though their efficacy has not been confirmed under Rwandan conditions. There is also a need to evaluate locally available plants which would be more affordable to local farmers.

1.2 Statement of the problem

Tuta absoluta invasion in Rwanda constitutes a threat to tomato production, the pest results in reduced yield and poor quality tomatoes as well as increased cost of production due to additional pesticide use. This leads to reduced farmers' income, decreased area under tomato production, and finally affects national revenue, food, and nutritional security. The control of *T. absoluta* in the country is mainly based on synthetic insecticides, often with higher doses and increased frequency of application. This result in health and environmental hazards, destruction of natural enemies and development of resistance to available active ingredients. Options for Integrated Pest Management (IPM) for *T. absoluta* control under Rwandan conditions have not been developed. Although EPNs and EPFs have been reported as effective against *T. absoluta*, no research has been done in Rwanda to evaluate the efficacy of native and exotic EPN isolates maintained in the Biological Control Laboratory of Rwanda Agriculture and Animal Resources Development Board (RAB) and commercial EPFs. Although *T. absoluta* control can be effectively achieved using plant extracts, the possibilities of using extracts from indigenous plants have not been explored in Rwanda to provide farmers with readily available, cheap and environmentally friendly alternatives.

1.3 Objectives

1.3.1 General objective

This study was designed to contribute to enhanced tomato productivity and quality in Rwanda through evaluation of entomopathogens and plant extracts as options for integrated pest management (IPM) of the tomato leaf miner (*Tuta absoluta* Meyrick).

1.3.2 Specific objectives

The specific objectives of this study were to determine the:

i) Potential of entomopathogenic nematode isolates (EPNs) from Rwanda to control *T. absoluta*
ii) Pathogenicity of selected commercial formulations of entomopathogenic fungi (EPFs) against *T. absoluta*
iii) Bioactivity of *Tithonia diversifolia*, *Tephrosia vogelii*, *Vernonia amygdalina*, and *Phytolacca dodecandra* plant extracts against *T. absoluta*
iv) Efficacy of selected entomopathogens and plant extracts against *T. absoluta* under field conditions in Rwanda
v) Effects of selected entomopathogens and plant extracts on tomato growth, yield and fruit quality.

1.4 Hypotheses

The present study tested the following null hypotheses:

i) Entomopathogenic nematode (EPN) isolates from Rwanda have no potential to control *T. absoluta*
ii) Selected commercial entomopathogenic fungi (EPFs) are not pathogenic against *T. absoluta*
iii) *Tithonia diversifolia, T. vogelii, V. amygdalina,* and *P. dodecandra* plant extracts are not bioactive against *T. absoluta*
iv) Selected entomopathogens and plant extracts are not effective against *T. absoluta* under field conditions in Rwanda
v) Selected entomopathogens and plant extracts have no significant effects on tomato growth, yield and fruit quality.

1.5 Justification

Tuta absoluta has been detected in Rwanda and the pest causes up to 100% yield loss in tomato, there is need for rapid response to cope with this threat. Increased hazards from synthetic pesticides justify the need to carry out the study on alternative management options against *T. absoluta*. This study was designed to contribute valuable knowledge on different options for management of *T. absoluta* in Rwanda.

The exploration of possibilities to use environmentally friendly options such as native EPN isolates, commercial formulations of EPFs, and indigenous plant extracts is an important contribution to sustainable management of *T. absoluta* in Rwanda. According to the Insecticide Resistance Action Committee (IRAC, 2011), local efficacy evaluation of the pest control strategies is very important and should be considered as the first step in any local *T. absoluta* IPM programme.

This study will serve as a baseline for further research on *T. absoluta* in Rwanda as it is the first scientific study, to the best of my knowledge. The methods developed and followed throughout this research will be adapted in other related researches aimed at finding sustainable solutions for management of *T. absoluta* and other invasive pests. The findings of this study will benefit stakeholders throughout the whole tomato value chain. Producers, consumers, processors, traders, and exporters will be able to get good returns on investment owing to high yield and quality of tomatoes hence high prices offered in the market. This will contribute to increased income and improved food and nutritional security.

1.6 Limitation of the study and assumptions

This study was restricted to four local EPN isolates plus two exotic EPN species, which were available and assessed in the Biological Control Laboratory of RAB. No other EPN species were introduced as they might not adapt to the local conditions. Three EPF strains commercially available in East Africa were selected based on the existing reports of their efficacy on other insect pests. The choice of plants, from which extracts were obtained and tested against *T. absoluta*, was limited to locally available plants as it was anticipated that the most effective plant extract was to be recommended for local farmers. This study assumed that the indigenous EPNs isolated from Rwandan agro-ecological conditions could have potential to control *T. absoluta* because they are more adapted to Rwandan conditions than exotic EPNs. It was also assumed that commercial formulations of EPFs and local plant extracts that are effective against other insect pests would also be effective against *T. absoluta.*

CHAPTER TWO

LITERATURE REVIEW

2.1 Overview of tomato production in Rwanda

In Rwanda, tomato production is dominated by small-scale farmers and mainly occurs under open field conditions. According to Clay and Turatsinze (2014), greenhouses occupied only 2.8 ha in 2013 and are used not only for tomato production, but also for other vegetables and flowers. Tomato production in Rwanda is done throughout the year in three seasons: A (short rain period from September to January), B (long rain period from February to June), and C (dry season, in marshland from June to September) (USAID, 2018).

About 240,000 households are involved in tomato production in Rwanda. Considering the total volume of horticultural crops produced in Rwanda in the year 2013, tomato occupied 28.4%, followed by onions (14.2%) and cabbages (12.8%) (Clay and Turatsinze, 2014). Most of the tomato growers are located in Kigali city and neighbouring districts such as Bugesera, Rwamagana, Kayonza, and Ngoma. Besides, huge tomato amounts are produced and sold from Rusizi, Huye, and Nyagatare Districts (Figure 2.1).

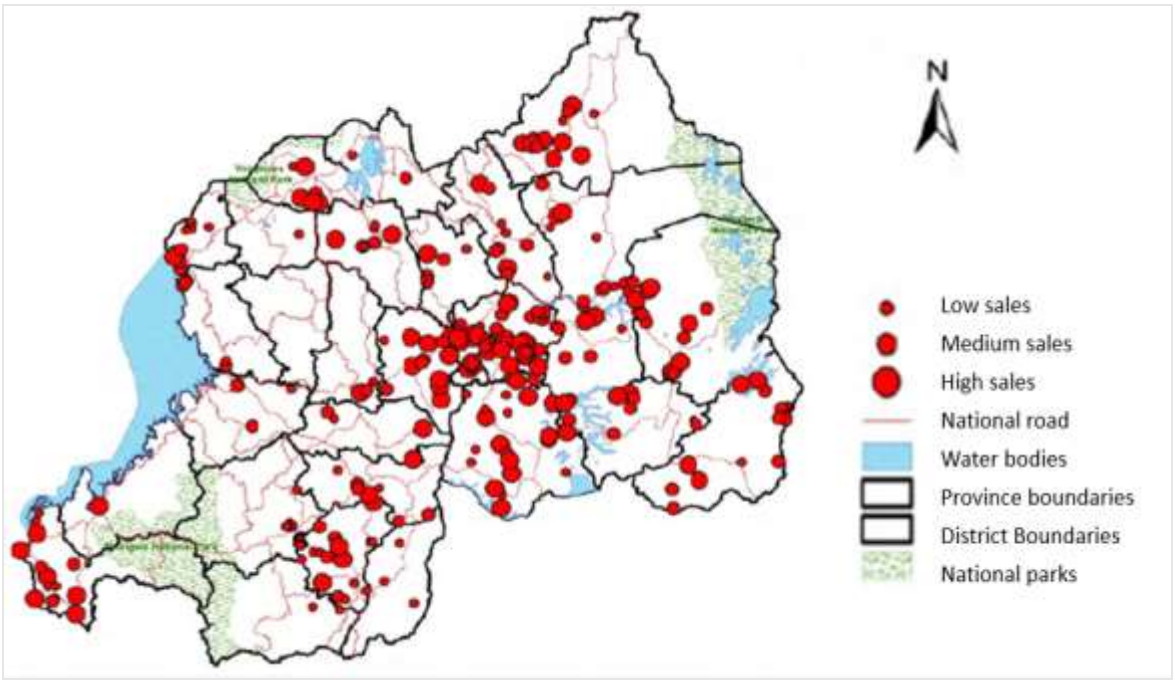

Figure 2.1 Tomato sales by district in Rwanda

Source: Clay and Turatsinze (2014)

According to USAID (2014), Rwanda is not self-sufficient in tomatoes and it is a net importer of tomatoes from neighbouring countries. About 20% to 30% of tomatoes produced in Rwanda are consumed by the farmers themselves while the remaining 70% to 80% are sold at the local markets. Data on the amount of tomatoes that are informally exported to neighbouring countries are unavailable. Almost all tomatoes produced are sold fresh without processing.

2.2 Constraints to tomato production in Rwanda

According to FAO (2019), the average world tomato yield was 37.6 t/ha in 2017, while it was only 8.6 t/ha in Rwanda during the same year. The low yield of tomato can be attributed to some production constraints faced by tomato farmers in Rwanda. A survey conducted by Rwanda Horticulture Development Authority (RHODA) in 2008 reported limited production skills, followed by pests and diseases as the main constraints faced by Rwandan horticultural farmers, including tomato producers (Figure 2.2).

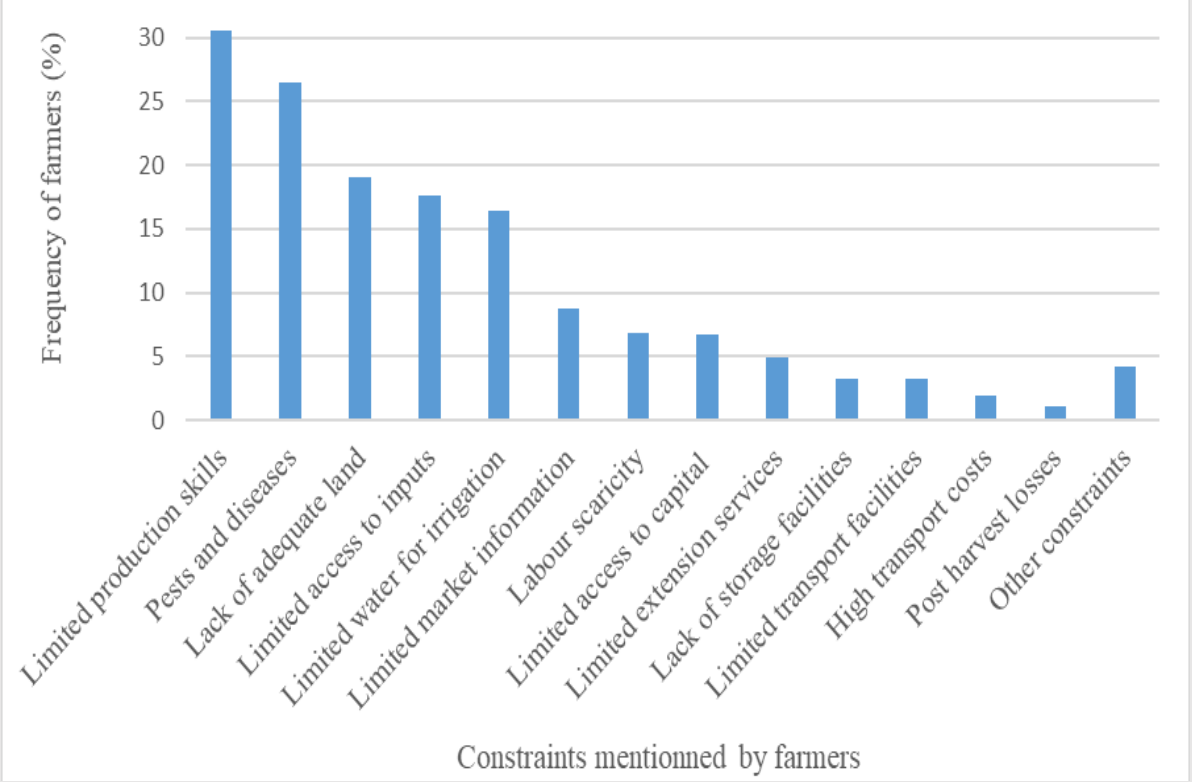

Figure 2.2 Constraints faced by horticultural farmers in Rwanda

Source: RHODA (2008)

Besides, USAID (2018) reported that prevalence of pests and diseases, limited adoption of modern inputs (seeds, fertilizers, and pesticides), and inadequate water for irrigation are among the factors limiting tomato production in Rwanda. It was observed that only 13%, 16%, and 18% of Rwandan vegetable farmers use improved seeds, pesticides, and inorganic fertilizers, respectively. Only 13% of small-scale vegetable farmers use irrigation.

The national integrated pest management (IPM) framework for Rwanda reported major diseases of tomato in the country to include: late blight (*Phytophthora infestans*), early blight (*Alternaria solani*), leaf moulds (*Fulvia fulva*), anthracnose (*Colletotrichum spp*.), powdery mildew (*Leveillula taurica*), septoria leaf spot (*Septoria lycopersici*), Fusarium wilt (*Fusarium oxysporum f. sp.lycopersici*), Verticillium wilt (*Verticillium dahliae*), damping off (*Pythium spp*. and *Rhizoctonia solani*), bacterial wilt (*Ralstonia solanacearum*), tomato yellow leaf curl virus, tomato mosaic virus, and blossom end rot. The major pests attacking tomato crop in Rwanda include: aphids (*Myzus persicae* and *Aphis gossypii*), whitefly (*Bemisia tabaci*), african spider mites (*Tetranychus spp.)*, root-knot nematodes (*Meloidogyne spp*.), bollworm (*Helicoverpa armigera)*, leafminer (*Liriomyza spp*.), and cutworm (*Agrotis spp*.) (REMA, 2011). The invasive tomato leaf miner, *Tuta absoluta*, was not mentioned in the above framework, because it was reported later in 2015 (FAO, 2015).

Although tomato is infected by many diseases in Rwanda, fungicides are only sprayed against late blight using mainly Mancozeb and Metalaxyl (REMA, 2011). To control insect pests, Rwandan farmers use different insecticides recommended by RAB, such as Abamectin, imidacloprid, Lambda-cyhalothrin, and Cypermethrin, among others.

2.3 *Tuta absoluta* as tomato pest

This section elaborates on taxonomy, history, biology, and ecology of *T. absoluta*. The damage and economic impact of *T. absoluta* are also discussed.

2.3.1 Taxonomy and history of *Tuta absoluta*

The invasive tomato leaf miner belongs to the domain Eucaryota, kingdom Metazoa, phylum Arthropoda, class Insecta, order Lepidoptera, family Gelechiidae, genus *Tuta*, and species *T. absoluta* (Meyrick) (Biondi *et al.*, 2018). It was named *Phthorimaea absoluta* by Meyrick in 1917 upon collection in Peru, and in 1994 it was renamed as *Tuta absoluta* by Povonly. It was acknowledged as a major pest since 1964 in Argentina from where it invaded the rest of South America (Desneux *et al*., 2010).

The invasion of the Latin American countries: Columbia, Bolivia, Peru, Panama, Venezuela, Brazil, Argentine, Chile, Ecuador, Paraguay, and Uruguay by *T. absoluta* took place during the period 1960 - 1980s (Giorgini *et al*., 2019). In the year 2006, this pest was reported in Spain and from there it spread to many other European countries, the Middle East and Northern Africa (Urbaneja *et al*., 2012). The period from 2006 to 2012 marked the drastic spread of *T. absoluta* in the Mediterranean basin (Garzia *et al*., 2012).

Algeria, Tunisia, Libya, Egypt, and Morocco were the first African countries to be invaded by *T. absoluta* during 2008 - 2009 (Zekeya *et al*., 2017; Mansour *et al*., 2018). It was detected in Sudan in 2010, Ethiopia in 2012, Kenya and Tanzania in 2014, Uganda and Rwanda in 2015, and South Africa in 2016. By 2018 the pest was established in 41 out of 55 African countries (Biondi *et al*., 2018; Mansour *et al*., 2018). Also, following the Turkey invasion in 2009, the pest quickly spread in almost all Southern West and Central Asian countries neighbouring China, the world's largest tomato producer (Mansour *et al*., 2018).

In only one decade following Spain invasion, *T. absoluta* spread drastically and the world tomato production area under its invasion increased from 3% to 60% (Biondi *et al*., 2018). This rapid spread of *T. absoluta* has been attributed to long distance trade of tomato fruits, in addition to greenhouse tomato production and nurseries that favoured overwintering and quick development of *T. absoluta* owing to the prevailing temperature inside these structures (Van Damme *et al*., 2015).

2.3.2 Biology and ecology of *Tuta absoluta*

The morphology of *T. absoluta* has many similarities with *Phthorimaea operculella*, a pest of potato belonging to the same family. This family includes more than 4000 insect species (Biondi *et al.*, 2018). The lifecycle of *T. absoluta* involves four developmental stages, namely, egg, larva, pupa, and adult (Figure 2.3).

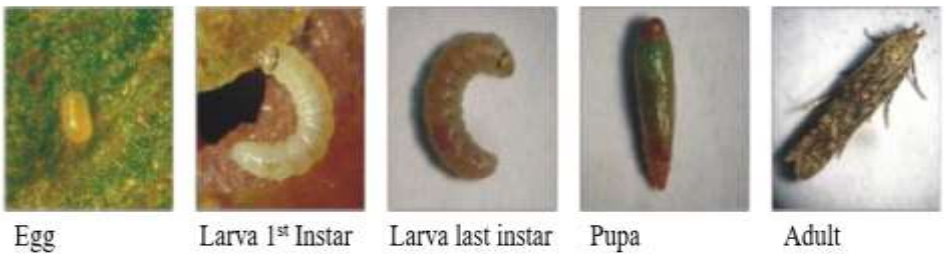

Figure 2.3 Developmental stages of *Tuta absoluta*

Source: Harizanova *et al*. (2009)

The life cycle of *T. absoluta* is mainly influenced by environmental conditions such as temperature and relative humidity. A period of 28.7 days has been reported by Garzia *et al*. (2012) under laboratory conditions of 25ºC temperature and 75% relative humidity (Garzia *et al*., 2012). Salama *et al*. (2014) further reported that developmental stages of *T. absoluta* also depends on temperature (Table 2.1). It has been observed that at 0ºC, half of *T. absoluta* population can survive up to 11, 13 and 18 days for larvae, pupae, and adults, respectively, though reproduction and development are hampered by low temperature (Cuthbertson *et al*., 2013; Biondi *et al*., 2018).

Table 2.1 Life cycle of *Tuta absoluta* under different temperature levels

Stages	Duration (Days ± SD)			
	At 15ºC	At 20ºC	At 25ºC	At 30ºC
Eggs	14.6 ± 0.9	8.7 ± 0.4	5.6 ± 0.2	3.7 ± 0.2
Larvae	32.0 ± 1.7	21.4 ± 1.1	14.6 ± 0.2	9.2 ± 0.2
Pupae	22.1 ± 0.5	16.6 ± 1.2	10.9 ± 0.3	6.5 ± 0.2
Longevity of male	21.6 ± 0.8	16.3 ± 0.4	10.1 ± 0.7	6.7 ± 0.2
Longevity of female	25.8 ± 1.9	21.3 ± 0.9	14.3 ± 0.2	11.9 ± 0.2
Life cycle of male	90.6 ±1.6	63.0 ± 7.8	41.2 ± 3.7	26.1 ± 0.3
Life cycle of female	94.6 ± 1.2	68.0 ± 1.6	45.4 ± 0.6	31.3 ± 0.4
Pre-oviposition	4.6 ± 0.5	4.0 ± 0.6	2.9 ± 0.8	2.3 ± 0.6

Source: Salama *et al*. (2014)

Tuta absoluta females mate up to six times in their life and only one mating is done per day and takes four to five hours. More than 70% of eggs are laid one week after mating in the late afternoon and 250 - 300 eggs can be laid by one female (Garzia *et al*., 2012). For oviposition and feeding, *T. absoluta* is attracted by volatile organic compounds released by the host plant (Proffit *et al*., 2011). *Tuta absoluta* females often lay eggs underneath the tomato leaves (73%), leaf veins and stems (21%), and less frequently on sepals (5%) and green fruits (1%) (Braham *et al*., 2012). They prefer laying on the leaves of the apical part of tomato plants, which could be due to tenderness of these leaves in comparison to middle and bottom parts (Cherif *et al*., 2013). These eggs are oval in shape and creamy white soon after they are laid but they later become yellow; and finally black before hatching (Salama *et al*., 2014).

Hatching takes place four days to six days after oviposition (Cuthbertson *et al*., 2013) and the larvae pierce the tomato leaves and fruits to feed upon mesophyll and internal fruit content, resulting in mines and galleries (Braham *et al*., 2012). Cherif *et al.* (2013) observed the highest number of larvae on the middle part of the tomato plant, which might be due to the highest number of leaves, and thus more food, as compared to the upper and lower parts. These larvae go through four instars and are creamy yellow immediately upon hatching but turn to greenish due to feeding, with dorsal end becoming reddish just before pupation (Salama *et al*., 2014). The fully developed larvae sometimes pupate on the host leaves but they generally drop to the ground on a silk thread and pupate in the soil (Queiroz *et al*., 2015).

Tuta absoluta pupae are cylindrical with greenish colour directly after pupation and brown before adult emergence. Males are narrow with a creamy abdomen, while females are bigger with a brown abdomen (Salama *et al*., 2014). The adults are nocturnal, expressing greater flight activity during the crepuscule and remain hidden amongst the leaves during the day (Queiroz *et al*., 2015). Overwintering of this pest as egg, pupal or adult was reported (EPPO, 2005). There is a wide variation in the number of generations for this pest per year and they were reported to be 12 in optimum conditions (Desneux *et al*., 2010).

2.3.3 Damage and economic impact of *Tuta absoluta*

Tuta absoluta can cause up to 100% yield losses (Desneux *et al*., 2010). The galleries formed in leaves by feeding within the mesophyll affect negatively the photosynthetic capacity of plants while the ones in stems disturb crop growth and development. The damages in fruits disqualify them from marketing and consumption (Urbaneja *et al*., 2012) (Figure 2.4). Also, these mines and galleries may serve as entry points for secondary pathogens that negatively affect crop growth and yield (Desneux *et al*., 2010).

Figure 2.4 Damage caused by *Tuta absoluta* on tomato crop. Larvae in leaf galleries (A), on shoots (B) and fruit (C); damaged tomato fruit (D) and crop (E).
Source: IRAC (2011)

Farmers in countries recently invaded by *T. absoluta* recorded a drastic increase in the cost of tomato production due to the added burden of controlling the pest using different options, which resulted in increased number of insecticide applications (Garzia *et al*., 2012; Biondi *et al*., 2018). Since the larvae enter the fruits through small holes, which are generally made under the sepals, detection of attacked fruits in the field is difficult (Biondi *et al*., 2018). This is the main reason for the non-invaded countries to restrict the importation of tomatoes from invaded countries (Desneux *et al*., 2011). Finally, this is a drawback in the whole tomato value chain, which hampers the export industry and has even caused some farmers to abandon tomato production (Zekeya *et al*., 2017; Biondi *et al*., 2018).

2.4 Management of *Tuta absoluta* in tomato

Different management strategies that have been in use worldwide against *T. absoluta* are reviewed under this section.

2.4.1 Cultural and physical control methods

Use of resistant cultivars is one of major IPM strategies, whereby resistance can be in form of tolerance, antibiosis, and antixenosis or a combination of all of them (Resende *et al*., 2006). Plant tolerance is the capacity to keep its production despite the pest attack (Sohrabi *et al*., 2017). Antixenosis is a limited plant colonisation by the pest for food, oviposition, and shelter, which may be due to plant's chemical, physical and morphological features (Resende *et al*., 2006); while antibiosis denotes the immediate harmful effect caused by a plant on pest's life and development (Sohrabi *et al*., 2017). Tomato variety preference by *T. absoluta* varies and this has been attributed to difference in leaf volatiles (Proffit *et al*., 2011; Cherif *et al*., 2013), glandular trichomes (Sohrabi *et al*., 2017), and allelochemical compounds known as acyl sugars (Resende *et al*., 2006). Other compounds knowns as zingiberene and 2-tridecanone were also reported to confer antixenosis form of tomato resistance to *T. absoluta* (Maluf *et al*., 2010; Sohrabi *et al*., 2017).

Resende *et al*. (2006) proved the ability of acyl sugars (AS) to confer resistance of tomato *to T. absoluta*. They observed that F2 plants, from intercrossing between *Lycopersicon pennellii* 'LA714'that has high content of AS, and *L. esculentum* 'TOM-584', were more resistant to *T. absoluta*. According to the same authors, these AS are a mixture of glucose and sucrose esters of fatty acids and are known to constitute around 90% of the type IV glandular trichomes secretions.

In Brazil, the transfer of resistance genes from wild species to cultivated tomato resulted in hybrids with high AS, which conferred resistance to *T. absoluta* to the same extent as in homozygous lines with high AS (Maluf *et al*., 2007). Maluf *et al*. (2010) reported that adequate resistance can be reached with intermediate AS contents. Oliveira *et al*. (2012) observed a positive relationship between resistance to *T. absoluta* and glandular trichomes density in different tomato lines evaluated. Although the glandular trichomes on tomato leaves were identified as a major source of resistance to *T. absoluta*, their transfer in commercial varieties negatively affected yield parameters (Guedes and Picanço, 2012).

Khederi *et al*. (2014) screened and reported that Ríogrande and King Ston tomato varieties are relatively resistant to *T. absoluta*. Sohrabi *et al*. (2017) evaluated the resistance of 11 tomato cultivars and observed that four of them: Berlina, Golsar, Poolad, and Zaman were more resistant to *T. absoluta*, compared to others, probably due to plenty of trichomes on their leaves. Thus, choice of tomato cultivars not suitable for oviposition by *T. absoluta* should be considered, where possible, in IPM programme against this pest. Plant resistance can also be improved by cultural practices such as reducing nitrogen application, but at levels that do not negatively affect plant growth, and optimum water application (Han *et al*., 2018). Mahamadi *et al*. (2017) reported reduced *T. absoluta* population density using vermicompost and humic fertilizer. Avoidance of alternative host plants, double door use in protected structures, removal of infested plant parts and old leaves are among the methods of controlling *T. absoluta* followed in Jordan (Al-Jboory *et al*., 2012). Destruction of crop residues, cultivation restrictions where the pest is detected, and physical isolation for greenhouses and warehouses are some of control methods used in Spain (Desneux *et al*., 2010).

In Tunisia, greenhouse equipped with insect-proof screens are used to restrict entrance of *T. absoluta* in these structures. For instance, Cherif *et al.* (2013) recorded 20 larvae on 75 leaves per week inside these contained structures, while the control had 40 larvae. Besides, removal of infested leaves and sprouts, and soil covering using plastic screens to inhibit emergence of adults from pupae that may be in soil, are also taken as promising strategies against *T. absoluta*. In Senegal, farmers opted not to plant tomato in middle of dry season when *T. absoluta* population is high; this considerably reduced *T. absoluta* damage on tomato crop. Besides, destruction of secondary hosts and volunteer plants is of great importance to reduce the proliferation *T. absoluta* (Mansour *et al*., 2018). Karadjova *et al*. (2013) opined that tomato packing materials are among the important means of entry of *T. absoluta* in new areas.

Therefore, measures have to be taken accordingly. Crop rotation excluding the succession of *Solanaceae* plants and farm sanitation by removing the tomato residue after harvest are also important cultural measures to manage this pest (Mansour *et al*., 2018).

2.4.2 Chemical control methods

Owing to the quick action of synthetic insecticides, chemical control is the primary option that is readily available for use in *T. absoluta* invaded fields (González-Cabrera *et al*., 2011). The control of *T. absoluta* was achieved using organophosphates, which were progressively substituted by pyrethroids in the 1970s. High efficacy was attained through alternation of cartap with pyrethroids and thiocyclam in the early 1980s. Abamectin, acylurea insect growth regulators, chlorfenapyr and tebufenozide were introduced during the 1990s (Lietti *et al*., 2005). Later in the 2000s, flubendiamide and chlorantraniliprole were included in *T. absoluta* management (Silva *et al*., 2011; Biondi *et al*., 2018). In their study, Mahmoud *et al*. (2014) and El-Ghany *et al*. (2016) obtained higher efficacy of lambda-cyhalothrin against *T. absoluta*. Collavino *et al*. (2008) also observed that imidacloprid was effective in controlling *T. absoluta*.

In Sub-Saharan Africa, chemical control is the main option used for *T. absoluta* management (Zekeya *et al*., 2017; Mansour *et al*., 2018). The Pesticide Sahelian Committee approved 11 synthetic insecticides on tomato crop. These include: organophosphates (chrolppyrfos-methyl, chrolppyrfos-ethyl, and profenofos), neonicotinoids (imidacloprid and acetamiprid), a carbamate (methonly), pyrethorids (deltamethrin, cypermethrin, lambda-cyhalothrin), and avermermectins (emamectin benzoate and abamectin) (Mansour *et al*., 2018). However, some of these chemicals are prohibited in Europe due to their harmful effects (Ali *et al*., 2018).

The ability of *T. absoluta* to develop resistant strains to frequently used insecticides has been recorded by many researchers and this resistance is accelerated by multiple applications of the same insecticide (Yalçın *et al*., 2015). For instance, the resistance of *T. absoluta* to deltamethrin, methamidophos, esfenvalerate, lambda-cyhalothrin, and mevinphos was observed in Chile (Salazar and Araya, 2001), while in Argentine the resistance to abamectin and deltamethrin was reported by Lietti *et al*. (2005). In Brazil, the resistance of *T. absoluta* to abamectin, cartap, methamidophos, and permethrin was reported by Siqueira *et al*. (2001). Roditakis *et al*. (2015) reported the resistance of *T. absoluta* to the diamide insecticides, chlorantraniliprole and flubendiamide, which were observed to possess higher efficacy

against the pests belonging to Lepidoptera order. In Iran, the resistance of *T. absoluta* to the pyrethroids deltamethrin, permethrin, and cypermethrin, and to organophosphates diazinon and chlorpyrifos was confirmed by Zibaee *et al*. (2018). Roditakis *et al*. (2018) also reported *T. absoluta* resistance to chlorantraniliprole (diamide), spinosad (spinosyn), indoxacarb (oxadiazine), and emamectin benzoate (avermectin).

In addition to alteration of target sites through mutations, resulting in a decreased sensitivity (Zibaee *et al*., 2018), the resistance of *T. absoluta* to synthetic insecticides is also attributed to enzymes like cytochrome P450s monooxygenase, glutathione-S-transferase, and esterase that act as detoxifying agents by breaking down or sequestrating the insecticide molecules before reaching the target sites (Reyes *et al.,* 2012). This phenomenon is among the main reasons for failure of many synthetic insecticides to control *T. absoluta* (Roditakis *et al*., 2015).

The use of synthetic insecticides has become unavoidable probably due to their simplicity to use and rapid action against the pest population. Hence, when they are used, a cautious frequent change of active ingredients should be followed to prevent selections of resistant genotypes of *T. absoluta*, which are difficult to managed (Braham *et al*., 2012). Moreover, overuse and/or misuse of synthetic insecticides often leads to water pollution, eradication of beneficial non targeted insects especially pollinators and natural enemies, human health hazards and several other adverse environmental effects (Macharia *et al*., 2009). Furthermore, the mine-feeding behaviour of *T. absoluta* larvae complicates and limits the effectiveness of relying solely on synthetic insecticides for its control and hence the necessity to investigate alternative options (Gebremariam, 2015).

2.4.3 Use of pheromones and traps

Pheromones are chemical substances released by organism to communicate with others in similar species (Witzgall *et al*., 2010). Specifically, sex pheromones are released by an organism to attract another of different sex within the same species for mating. The female pheromones of *T. absoluta* consist of two constituents: (3E, 8Z, 11Z)-3,8,11-tetradecatrien-1-yl acetate or TDTA and (3E, 8Z)-3,8- tetradecadien-l-yl acetate or TDDA (Figure 2.5). These two components represent 90% and 10%, respectively, of the volatile substance found in sex gland of *T. absoluta* femalesHowever, it has been found that pheronome traps loaded with only TDTA (100 μg) can work successfully (Megido *et al*., 2013).

Figure 2.5 Two constituents of *Tuta absoluta* female sex pheromone: (a) (3E, 8Z, 11Z)-3,8,11-tetradecatrien-1-yl acetate or TDTA and (b) (3E, 8Z)-3,8- tetradecadien-1-yl acetate or TDDA.

Source: Megido *et al.* (2013)

Sex pheromones have been used widely and successfully to detect and monitor insect pests' population. They are often used in combination with traps, which capture the males when they are coming for mating (Goftishu *et al*., 2014). Mass trapping (attract-and-kill) and mating disruptions are two main ways of using sex pheromones to control insect pests (Witzgall *et al*., 2010). Most traps used for monitoring *T. absoluta* have a triangular shape, often called Delta traps, and consist of a lure hanged above a gummy removable panel that catches male adults (Hassan and Al-Zaidi, 2010). Traps fixed at a height of 60 cm captured higher number of males despite the crop growth stage (Megido *et al*., 2013).

Pheromones can thus be used to monitor and decide the right time to intervene against a particular pest considering the number of insects trapped, which usually coincide with larval damaging activity (Goftishu *et al*., 2014). In this way, they facilitate the concept of IPM that is based on regular pest checks for planning control measures (Witzgall *et al*., 2010). For monitoring purposes, two pheromone traps can be fixed per ha and action should be taken as soon as one trap has captured 50 males per week (Mansour *et al*., 2018). Bolckmans (2009) recommended 10 to 20 traps/ha in greenhouse nursery as a strategy of monitoring and mass-trapping of *T. absoluta* at the same time.

According to USDA APHIS (2011), mass trapping using water traps (plastic container having water and a lure fixed conveniently above the water) are preferred because they can trap many males compared to Delta traps. A little vegetable oil or soap can be added to the water to reduce its surface tension, which lowers the ability of captured insects to escape. Besides, sticky traps (rolls with gummy surface imbedded with pheromones) can be used for mass trapping of *T. absoluta* males. Rolls of yellow colour concurrently capture aphids and whiteflies; but they were reported to also trap natural enemies. Thus, in cultivation systems depending on beneficial insects, white rolls should be used (Hassan and Al-Zaidi, 2010).

In a greenhouse experiment, Filho *et al*. (2000) observed that about 233 males of *T. absoluta* could be trapped daily by one pheromone trap and this significantly reduced female mating and decreased the pest population. From their study, Harbi *et al*. (2012) concluded that one pheromone trap per 3500 m^2 greenhouse covered with an insect-proof net can reduce losses caused by *T. absoluta* on tomato. However, in Tunisia, Cherif *et al*. (2018) observed that two pheromone traps were more effective per 500 m^2 greenhouse compared to one or three traps; while Cocco *et al*. (2012) reported a failure of one, two, and three pheromone traps to reduce *T. absoluta* leaf and fruit damages in a greenhouse of 250 m^2. This shows that relying only on pheromones traps cannot guarantee effective control of *T. absoluta* because contradicting results have been reported. Possibilities of field application of lure and kill substances, combined or used separately, were also explored. For instance, Hassan *et al*. (2010) and Witzgall *et al*. (2010) reported that a combination of 3% cypermethrin and 0.3% sex pheromone can be sprayed in field against *T. absoluta* as an attract and kill strategy. In Spain, mass trapping and then killing of *T. absoluta* adults using authorized chemicals or microbiological products are followed among other techniques (Desneux *et al*., 2010).

Mating disruption techniques is achieved by applying high amount of a synthetic pheromone that create confusion among male adults so that mating does not take place (Cocco *et al*., 2013). It has been reported that at higher infestation of *T. absoluta*, this technique was effective and resulted in reduced pest population, reduced leaf and fruit damagesin well isolated greenhouses to prevent entrance of mated females (Vacas *et al*., 2011; Cocco *et al*., 2013). Nevertheless, contradicting results have been obtained in greenhouses with lower *T. absoluta* infestation (Vacas *et al*., 2011) and under open-field conditions (Filho *et al*., 2000). Besides, since higher amounts of pheromones are used (up to 60 g/ha), the cost of pheromones limits the wide use of this technique in IPM of *T. absoluta* (Cocco *et al*., 2013).

Since pheromones are environmentally friendly, they are recommended when the pest density is low for sustainable pest management instead of synthetic pesticides (Witzgall *et al*., 2010). However, using pheromones as a pest control strategy has generally low success despite a large number of males, which can be trapped (Cocco *et al.*, 2013; Gebramariam, 2015). Besides, Megido *et al*. (2013) reported the limitations of pheromone traps against *T. absoluta* because females of this pest can reproduce parthenogenetically. Desneux *et al*. (2010) suggested that mass trapping should be combined with insecticides to be operational.

In their study, Cocco *et al*. (2012) observed that light traps were able to significantly reduce leaf damage by *T. absoluta* and population density of the pest. By using one light trap per 350 m^2 inside a greenhouse, they recorded 267 adults of *T. absoluta*, both males and females, per trap. Leaf and fruit infestations were lower as compared to untreated greenhouse. Light traps are now being used in different parts of the world as a component of *T. absoluta* management (Biondi *et al*., 2018). Megido *et al.* (2013) recommended that pheromones traps should be combined with light traps so that both male and female adults are captured. Besides, a new generation of pheromone traps combining a pheromone lure, a water trap and a particular light wave length that incites and attracts *T. absoluta* adults were designed by Russel IPM Ltd. This permits trapping of both males and females, increasing their effectiveness up to 300% as compared to the usual pheromone traps (Hassan and Al-Zaidi, 2010; USDA APHIS, 2011). However, the new generation pheromone traps have not been widely adopted.

2.4.4 Use of entomophagous insects

The currently known parasitoids of *T. absoluta* belong to 20 species of Hymenoptera order and among them, *Pseudoapanteles dignus* and *Dineulophus phthorimaeae* perform 50% of natural parasitism. They have a potential of being used in biological control of *T. absoluta* in the area of origin and might also exhibit promising results in newly invaded areas (Luna *et al*., 2015). Several species of *Trichogramma* genus were identified as efficient in controlling *T. absoluta* (Cabello *et al*., 2009). In Tunisia, Zouba and Mahjoubi (2017) recorded 75.5% reduction in damage caused by *T. absoluta* in a greenhouse by releasing 40 adults of *Trichogramma cacoeciae* per tomato plant every three to four days. Nevertheless, limited suitability of *T. absoluta* to *Trichogramma* species was observed and this results in low field establishment of these natural enemies, which requires multiple releases and makes them less economically viable in management of *T. absoluta* (Chailleux *et al*., 2013).

Some predators like *Nesidiocoris tenuis, Macrolophus pygmaeus,* and *Dicyphus marrocannus* were observed preying on *T. absoluta* directly after its invasion in Mediterranean region. Laboratory assays confirmed that they are highly effective against this pest and they are currently used through inoculative release or conservation to provide a sustainable solution for management of this pest in Europe (Urbaneja *et al*., 2012). These omnivorous mird predators attack and feed on eggs and young larvae of *T. absoluta*, significantly reducing its population (Mollá *et al*., 2011). However, it was reported that they can have a negative effect on tomato crop. For instance *N. tenuis* adults and nymphs can feed and cause necrotic rings

on tomato plants and fruits, especially when they are many and do not find sufficient eggs and larvae of *T. absoluta* to feed upon. This leads to compromised tomato plant growth, yield, and fruit quality (Castañé *et al*., 2011; Biondi *et al.*, 2018). On the other hand, insignificant negative effects have been observed with *M. pygmaeus* and *D. marrocannus*, which were also reported to simultaneously provide effective control of whitefly, thrips, mites, and aphids among other pests (Castañé *et al*., 2011).

Queiroz *et al.* (2015) studied the functional response of three predatory pirate bugs: *Amphiareus constrictus* (Stal), *Blaptostethus pallescens* Poppius, and *Orius tristicolor* (White) (Hemiptera: Anthocoridae), which were observed to prey naturally on *T. absoluta* eggs in Brazil; they observed that these three predators are effective in regulating the population of this pest.

To develop a promising IPM programme based on using parasitoids and predators, success is higher with indigenous natural enemies, which are established in a particular environment (Luna *et al*., 2015). This emphasizes the necessity to carry out investigations in local conditions in order to identify beneficial insects that can be adopted in biological control of *T. absoluta*. Desneux *et al*. (2011) and Zappalà *et al*. (2012) reviewed a long list of predators and parasitoids, which have the potential of being used against *T. absoluta*. To conserve such effective natural enemies (conservation biological control), selective insecticides and alternatives to synthetic pesticides are needed (Zappalà *et al*., 2012). Other approaches of biological control, namely augmentative biological control (using mass-multiplied native natural enemies) and classical biological control (using exotic natural enemies) were used successfully in greenhouses; nevertheless, they are no reports on their successful use in open field conditions (Han *et al*., 2019).

2.4.5 Use of entomopathogens and bio-insecticides

Management of pests with entomopathogens and bio-insecticides is mainly aimed at reducing the use of broad-spectrum pesticides and thus conserving the environment. This allows for exploitation of the activity of natural enemies for a particular pest (Luna *et al*., 2015). Since the biocontrol agents do not disturb the ecosystem equilibrium, the pest outbreak is unlikely to happen (Mantzoukas and Eliopoulos, 2020). Different options that can be used to control *T. absoluta* were worked on by different researchers and are discussed in the following sections.

Entomopathogenic nematodes

Entomopathogenic nematodes (EPNs), specifically *Steinernema* and *Heterorhabditis* genera have long been used to control diverse crop pests under field conditions. They were easily adopted because they have a short life cycle and are easy to multiply (Georgis *et al*., 2006; Jaffuel *et al*., 2020). In the early stage of their life, these EPNs live as parasites and become free-living in their third stage as infective juveniles (IJs). The IJs are responsible for searching and invading the host; they utilise the stored food and do not feed until they get the host. They can survive for several months in the absence of a host. Upon invading the host, the IJs release in the host's hemolymph the associated symbiotic bacteria: *Photorhabdus* spp. or *Xenorhabdus* spp. for *Steirnernema* and *Heterorhabditis*, respectively (Stock, 2015; Jaffuel *et al*., 2020). Once released, these bacteria multiply and kill the host in 24 to 72 h. They also release antibiotics, bacteriocins, and antimicrobials to protect the killed host against other invaders so that the IJs can freely feed on it and also multiply (Griffin, 2012).

Infective juveniles use different pest scavenging strategies: most *Heterorhabditis* species are associated with cruiser strategy, a few including *Steirnernema carpocapsae* use ambusher strategy while *S. feltiae* uses an intermediate pest scavenging strategy. Through cruiser behaviour, the IJs move around to look for their hosts while the IJs with ambusher behaviour remain in one place (Lewis *et al*., 2006; Stock, 2015). Lacey *et al*. (2015) reported that in 2015, at least 13 companies were producing EPNs at the commercial level. These companies were distributed in America, Europe, and Asia. The species which were being used commercially included: *S. feltiae*, *S. carpocapsae, S. longicaudum, S. riobrave*, *S. glaseri*, *S. kraussei*, *S. scapterisci*, *S. kushidai*, *Heterorhabditis bacteriophora, H. zealandica, H. indica, H. megidis,* and *H. marelata*. This indicates the popularity of EPNs all over the world.

Initially, EPNs were used against the soil pests; however, their use has now extended to the insects living in cryptic habitats such as in tree barks and mines, including *T. absoluta* (Garcia-del-Pino *et al*., 2013). Different studies revealed that EPNs can be used effectively against *T. absoluta*. For instance, Batalla-Carrera *et al*. (2010) found that the dose of 25 IJs/cm^2 resulted in larval mortality of 78.6%, 85.7% and 100% using *Heterorhabditis bacteriophora*, *Steinernema carpocapsae,* and *S. feltiae*, respectively, while pupal mortality was only 10%, 6.7% and 3.3%, respectively. A dose of 60 IJs/cm^2 (equivalent to 1000 IJs/mL), controlled *T. absoluta* larvae inside the leaf galleries with mortality rates of 76.3%, 88.6% and 92% using *H. bacteriophora*, *S. carpocapsae*, and *S. feltiae*, respectively.

Similarly, Youssef (2015) observed that three dosages of 250, 500 and 1000 IJs/mL for *S. carpocapsae* caused *T. absoluta* larval mortality of 80%, 100% and 100% respectively; while mortality of three days old pupae was only 13.3%, 20% and 26.7%, respectively.

Late larval instars (3rd and 4th) of *T. absoluta* were observed to be the most susceptible to EPNs (Batalla-Carrera *et al*., 2010; Van Damme *et al*., 2016). Batalla-Carrera *et al*. (2010) observed that among the adults of *T. absoluta*, which emerged from pupae surviving the treatment by different EPN species, 6.7% were infected by *H. bacteriophora* while 40% were infected by *S. fertiae* and *S. carpocapsae.* A similar trend was also reported by other researchers (Garcia-del-Pino *et al*., 2013; Youssef, 2015). Among different species of EPN, the most virulent against *T. absoluta* was observed to be *S. feltiae* (Batalla-Carrera *et al*., 2010; Gözel and Kasap, 2015; Van Damme *et al*., 2016). Susurluk and Ehlers (2008) reported that EPNs can persist for about one year in field crops. EPNs can attack and develop in hosts killed by different pesticides and this permits survival, reproductivity, and persistence of natural and applied EPN population (Garcia-del-Pino *et al*., 2013).

Factors affecting efficacy of EPNs include: species or isolates, application concentration, and the environment. Since EPNs are very susceptible to desiccation and UV light (Makirita *et al*., 2020), it is recommended to apply them during cool hours of the day, like early morning or evening (Lacey *et al*., 2015). Furthermore, the effectiveness of EPNs was observed to be high with strains isolated in pest's environment owing to their adaptability to these conditions (Půža, 2015). Thus, before using EPNs in *T. absoluta* management, different strains should be tested to obtain the most effective in a given environment. In fact, according to Georgis *et al.* (2006) and Stock (2015), the biology and behaviour of the EPN, the target host and the environment where it will be applied should be considered when designing a control programme using EPNs.

Entomopathogenic fungi

Commercial Entomopathogenic fungi (EPFs) development as bio-pesticides has been focused on species in the order Hypocreales, to which *Beauveria bassiana* and *Metarhizium anisopliae* belong (Lacey and Shapiro-Ilan, 2008). Among 171 mycoinsecticides and mycoacaricides that have been in use worldwide since the 1960s, 68% were based on *B. bassiana* and *M. anisopliae* (Faria and Wraight, 2007). These products were in use in South America (42.7%), Central America (7%), North America (20.5%), Asia (12.3%), Europe (12.3%), Africa (2.9%) and Oceania (2.3%).

Beauveria bassiana is a fungus that lives naturally in soil and has been used as a biological control agent against many crop pests (Stafford and Allan, 2010). It was first isolated by Elie Metchnikoff and named as *Entomophora anisopliae* in the 1880s. *Metarhizium anisopliae* is also a widely distributed soil-borne fungus that was first isolated by Elie Metchnikoff and named *Metarhizium anisopliae* (Lord, 2005). Successful pest control with EPFs is realised when enough propagules come into contact with a host and when conditions are appropriate (high humidity) for fungal development (Lacey *et al*., 2015). The spores of the fungi, when in contact with the insect host, germinate and grow inside the body and finally kill it resulting in the production of new spores (Reda and Hatem, 2012; Mantzoukas and Eliopoulos, 2020).

The virulence of EPFs varies with strains and is conveyed by plenty of spore-bound proteases, efficiency in production and release of exoenzymes when they penetrate the host's cuticle, and efficiency the production of toxins during host colonisation (Lacey *et al*., 2015). Evaluation of different fungi strains is thus important to find out the most virulent that can provide effective control of a particular pest. Lacey *et al*. (2015) emphasized that in addition to fungi selection in laboratory bioassays where conditions seem to be optimum for their development, selection should also be advanced to field conditions to find out whether they can perform well in the environment where the targeted pest occurs.

Entomopathogenic fungi have been reported to be effective against diverse pests (Contreras *et al.*, 2014). Youssef (2015) observed that the doses of 10^8, 10^9 and 10^{10} spores/mL resulted in *T. absoluta* larvae (4^{th} instar) mortality rates of 86.7%, 100%, and 100% with *B. bassiana,* 76.7%, 83.3%, and 93.3% with *M. anisopliae*, respectively. Also, mortality rates of 81.5%, 79.1%, 69.6% for *B. bassiana* and 89.3%, 81.4%, 79.0% for *M. anisopliae*, respectively, were obtained for the emerged *T. absoluta* adults from treated pupae. The pupae were less infected as the mortality rates of 13%, 13.3% and 20% for *B. bassiana* and 23.3%, 6.7% and 10% for *M. anisopliae* were recorded for 3 days old pupae. The mortality rates decreased with the age of pupae. However, Contreras *et al*. (2014) reported that *M. anisopliae* was effective against the subterranean life stage (pupa) of *T. absoluta.*

Mortality rates up to 60% and 83% for *T. absoluta* larvae inside and outside the galleries, respectively, were also recorded by El-Ghany *et al*. (2016) with *B. bassiana* at the concentration of 10^9 colony forming units (CFU)/mL. Shalaby *et al*. (2013) observed a linear relationship between mortality rates of *T. absoluta* and concentrations of *B. bassiana* and *M. anisopliae*, which were more effective against eggs and newly hatched larvae.

El-Kichaoui *et al*. (2016) obtained up to 95% mortality of *T. absoluta* larvae mortality using concentrations of 2.5 x 10^7 spores/mL for *B. bassiana* fungus isolated from Gaza Strip. All the above-mentioned examples of the efficacy of EPFs against *T. absoluta* show that they can be effectively used in the management of *T. absoluta*. However, they should first be evaluated in specific conditions where they are to be used.

Bacillus thuringiensis

The formulations based on *Bacillus thuringiensis* contain mainly spores and toxins contrary to most of the other microbial control agents like fungi, nematodes, and viruses that contain the whole organisms. The crystal protein toxins of *B. thuringiensis* produced during sporulation confers insecticidal properties. These toxins mainly act as stomach poisons and kill insects through disruption of osmotic balance in the midgut epithelium, which leads to interruption of feeding and gut paralysis, and eventually death (Garczynski and Siegel, 2007). *Bacillus thuringiensis* is the most commonly used bacteria species in biological control of various pests (Ruiu *et al*., 2015).

The bio-insecticides based on *B. thuringiensis* have several advantages: absence harmful effect to human and other vertebrates (IPCS, 1999), excellent compatibility with natural enemies (González-Cabrera *et al.*, 2011) and possible use in case the treatment is needed immediately before harvest (Charles *et al*., 2013). They are also effectively used when insect resistance to frequently used pesticides is observed (Mollá *et al*., 2011). Different strains of *B. thuringiensis*, such as *B. thuringiensis* subsp. *kurstaki* and *B. thuringiensis* subsp. *aizawai*, have been observed to be effective against Lepidoptera pest species (Ruiu *et al*., 2015).

Different studies (Sabour and Nayera, 2014; El-Ghany *et al*., 2018) reported that *T. absoluta* can be effectively controlled using *B. thuringiensis*. González-Cabrera *et al*. (2011) reported that the most frequently used *B. thuringiensis* based formulations were able to significantly reduce the negative effects of *T. absoluta* on tomato crop. Mollá *et al*. (2011) observed that tomato plants treated with *B. thuringiensis* had no fruit infestation, probably due to high mortality of young larvae of *T. absoluta* which are mainly responsible for fruit damage (Desneux *et al*., 2010). Studies conducted in Spain revealed that first-instar larvae of *T. absoluta* were the most susceptible to *B. thuringiensis* (González-Cabrera *et al*., 2011). Sabbour and Nayera (2014) evaluated the effect of *B. thuringiensis Diple* (2X), *B. thuringiensis kurstaki* HD-73, and *B. thuringiensis kurstaki* HD-234 on *T. absoluta* and confirmed their effectiveness in controlling this pest.

In field experiments, Magda and Moharam (2015) studied seven isolates of *B. thuringiensis* and observed that they all significantly reduced *T. absoluta* infestation as compared to control. Higher tomato yield was also obtained in plots treated with *B. thuringiensis* isolates as compared to control plots. El-Ghany *et al*. (2018) also reported that *B. thuringiensis* subsp. *kurstaki* reduced *T. absoluta* population up to 71% - 91% in greenhouse experiment.

According to Amizadeh *et al.* (2015), parallel use of *B. thuringiensis* and chemical insecticides is not recommended for *T. absoluta* control due to antagonism effects. Though the efficacy of *B. thuringiensis* was observed against about 3000 insect species, limited studies have been conducted on susceptibility of *T. absoluta* to *B. thuringiensis* and these studies are done mainly in the pest's area of origin (Jallow *et al*., 2019). Although bio-insecticides based on *B. thuringiensis* are environmentally friendly, different authors have reported that some insects could develop resistance to these products (Gassmann *et al*., 2009). Mollá *et al*. (2011) recommended that, as an anti-resistance development strategy, *B. thuringiensis* based formulations should be used in alternation with other pest control agents or with other different subspecies of *B. thuringiensis* with varied toxin profiles.

Spinosad

Spinosad is a bio-insecticide composed by spinonyns A and D (Figure 2.6) which are the fermentation products of *Saccharopolyspora spinose*, a soil actinomycete. It is a contact and stomach poison, and acts by disrupting the nicotinic acetylcholine and gamma-aminobutyric acid receptors of insects (Kirst, 2010).

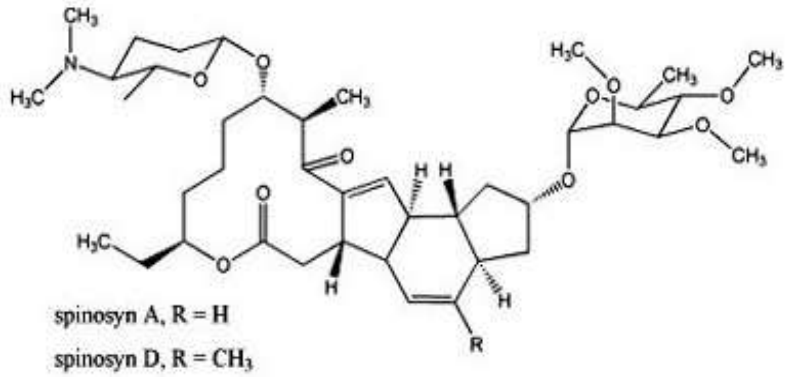

Figure 2.6 Structure of spinosyn A and spinosyn D

Source: Kirt (2010)

Spinosad was registered in over 30 countries worldwide and had been used on more than 150 crops by the year 2000 due to its low toxicity to natural enemies, aquatic ecosystems, mammals and humans (Cleveland *et al*., 2002). Higher efficacy of spinosad was obtained on pests belonging to Diptera and Lepidoptera orders (Abdelgaleil *et al.*, 2015).

In Brazil, spinosad was among the pesticides mostly used in management of *T. absoluta* during the year 2014 and was recommended in organic production (Campos *et al*., 2014). In their laboratory study, Hashemitassuji *et al*. (2015) observed higher mortality of the first, second and third instar larvae of *T. absoluta* using spinosad and this was significantly higher than *B. thuringiensis var. kurstaki*. Higher mortality was also obtained with combination of spinosad and *B. thuringiensis* compared to when there were used separately. Abdelgaleil *et al.* (2015), Bratu *et al*. (2015) and El-Ghany *et al.* (2018) have also reported high efficacy of spinosad on *T. absoluta* control.

However, several other studies have reported the failure of spinosad to effectively control *T. absoluta* in Chile, Brazil, Turkey and other places due to quick development of resistant genotypes (Reyes *et al*., 2012; Yalçın *et al*., 2015). Also, it has been reported that spinosad is harmful to different parasitoids and predators belonging to Hymenoptera that are among the natural enemies of *T. absoluta* and other important tomato pests such as whitefly (Biondi *et al*., 2018). Campos *et al*. (2014) recommended that the use of spinosad against *T. absoluta* should be avoided especially in newly invaded areas.

2.5 Potentials of indigenous plant extracts to control *Tuta absoluta*

Use of plant extracts is a good component of IPM as they play an important role in conservation of natural enemies and do not cause any harmful effect to humans and non-targeted organisms, which have been among the key problems associated with synthetic insecticides (Moshi and Matoju, 2017; Isman, 2020). Botanical insecticides have shown to be effective against several insects of different families (Nilahyane *et al*., 2012; Ghanim and Ghani, 2014; Isman, 2020). For instance, Salama and Shehata (2017) obtained 76.7%, 63.3%, 43.3% and 26.7% mortality of the first instar larvae of *T. absoluta* six days after treating with ethanolic extract of garlic (*Allium sativum*), using the concentrations of 2000, 1000, 500 and 250 ppm, respectively. Nilahyane *et al*. (2012) also reported 95% *T. absoluta* larval mortality using ethanolic extract of *Thymus vulgaris* at the concentration of 46.7 g/L.

Higher mortality was also recorded using ethanolic extracts of clove (*Syzygium aromaticum*), peppermint (*Mentha spicata*) and eucalyptus (*Eucalyptus camaldulensis*) (Salama and Shehata, 2017) and hexane extracts of *Acmella oleracea* (Moreno *et al*., 2011). Furthermore, Ghanim and Ghani (2014) achieved higher mortality of the second instar *T. absoluta* larvae using aqueous extracts of geranium, chinaberry, garlic, and onion in laboratory and greenhouse experiments.

Higher efficacy was also obtained with plants belonging to Piperaceae family (Brito *et al*., 2015). Up to 98.3% mortality of the second instar *T. absoluta* larvae, inside leaf galleries, was reported by Abdel-Baky and Al-Soqeer (2017) using extracts from seeds of Jojoba plant (*Simmondsia chinensis*). Moreover, Shiberu and Getu (2017) reported that crude extracts of *Azadirachta indica, Cymbopogon citratus,* and *Allium sativum,* used at the concentration of 10%, resulted in *T. absoluta* larval mortality rates of 98%, 97% and 95%, respectively, under laboratory conditions. In field, the same authors obtained up to 66.5% mortality of *T. absoluta* larvae using *A. indica.*

Azadirachtin is a natural tetranortriterpenoid extracted from the seeds of neem tree (*Azadirachta indica* Juss) and the fruits of chinaberry (*Melia azedarach*) (Mordue and Alasdair, 2000). It is one of the pesticides recommended against *T. absoluta* in Mediterranean region (Giorgini *et al.,* 2019). Azadirachtin was reported to exhibit little harmful effects on *Macrolophus pygmaeus* and *Nesidiocoris tenuis* (Hemiptera: Miridae), which are common generalist predators that possess ability to simultaneously control *T. absoluta* and other important pests including spider mites, thrips, whiteflies, leafminers, and leafhoppers (Arnó and Gabarra, 2011). Durmuġoġlu *et al*. (2011) reported higher efficacy of azadirachtin against *T. absoluta* under laboratory conditions. Similar results were obtained by Gonçalves-Gervásio and Vendramim (2007) who recorded 52.4% - 95.5% mortality of *T. absoluta* larva using neem seeds extract. El-Ghany *et al*. (2018) also reported 70% - 83% reduction in *T. absoluta* population using azadirachtin.

All the above-mentioned studies demonstrate that potential exists to manage *T. absoluta* using plant extracts. They are readily available and have more than one active ingredient that work synergistically and complicate the development of insect resistance against them (Braham *et al*., 2012). Furthermore, developing countries like Rwanda, which are rich in endemic plant diversity may have an added advantage by using plant extracts in IPM (Isman, 2020). Locally available plants such as *Tephrosia vogelii, Tithonia diversifolia, Phytolacca dodecandra*, and

Vernonia amygdalina have been reported to possess insecticidal properties. However, extracts from these plants have never been evaluated on *T. absoluta* in Rwanda.

Tephrosia vogelii has been in use against many field and storage pests (Boeke *et al*., 2004; Moshi and Matoju, 2017). Ogendo *et al*. (2004) observed that the ground powder of *T. vogelii* used at 0.05% weight/volume (w/v) was equally effective as actellic super 2% dust in reducing insect damage in stored maize grains. *Tithonia diversifolia* extracts were reported to suppress the population of various insects under field conditions on cowpea other crops (Ambrósio *et al*., 2008; Mkenda *et al*., 2015). Olufemi *et al*. (2015) obtained 100% mortality of four insect pests of honeybees: *Crematogaster lineolate*, *Aethina tumida*, *Achroia grisella*, and *Galleria mellonella* using N-hexane extracts of *T. diversifolia*. At concentrations of 1.0% and 1.5%, hot water leaf extract of *T. diversifolia* had similar efficacy permethrin in deterring oviposition in female *Sitophilus zeamais* (Onekutu *et al*., 2015).

Phytolacca dodecandra has been used by farmers around Lake Victoria to treat crops against various pests in field and storage (Mihale *et al*., 2009). Ethanolic extracts of *P. dodecandra* leaves caused mortality of 98% for *Sitophilus zeamais* and 99% for *Tribolium castaneum* with a dose of 150 mg/mL after three days (Qwarse *et al*., 2016). *Vernonia amygdalina* was reported to be toxic against flea beetles on Okra, bean aphids and weevils (Kawuki *et al.,* 2005; Adeniyi *et al*., 2010). Thus, it is worthy to evaluate these locally available plants on *T. absoluta* so that they can contribute to the sustainable management of this pest in Rwanda.

2.6 Effects of entomothogens and plant extracts on plant growth, yield and quality

Limited research has been conducted on effects of entomopathogenic nematodes and entomopathogenic fungi on plant growth, yield and quality parameters. Since these biorational control agents have been observed to significantly reduce the population of pests and their damage on different crops (Gözel and Kasap, 2015; Nilahyane *et al*., 2012; Youssef, 2015), they would also have effects on their growth, yield and quality of harvested produce.

Through endophytic activity, spores of the entomopathogenic fungi *Beauveria bassiana* also enter in plant tissues where they get protection against adverse environmental conditions, such as UV rays and dessication, and can persist for many months (Klieber and Reineke, 2016; Nishi *et al*., 2020). By colonising vascular tissues, these spores would be expected to impede the normal plant growth. However, different researchers reported that *B. bassiana* does not hamper plant growth (Klieber and Reineke, 2016; Allegrucci *et al*., 2017).

Elena *et al*. (2011) reported that *Metarhizium anisopliae* promotes plant growth as they obtained significant effects for tomato plant height, root length, shoot and root dry weight using three isolates of *M. anisopliae*. The effect of *M. anisopliae* on tomato plant growth varied among isolates and doses. Root and shoot dry weights increased up to 205% and 332%, respectively, as compared to untreated control plants. Maniania *et al*. (2003) obtained non-significantly different onion yield between plots applied with *M. anisopliae* and plots applied with dimethoate.

So far, there are no reports available on any negative effect of entomopathogenic nematodes (EPNs) on plant growth, yield and quality. Yan *et al*. (2012) obtained higher cabbage yield using the EPNs *Steinernema carpocapsae* All and *Heterorhabditis indica* LN2 to control striped flea beetle, *Phyllotreta striolata* (Coleoptera: Chrysomelidae) as compared to azadirachtin and controls (rotenone EC 2.5 % and water). Mutegi *et al.* (2017) obtained the yields of 9.8 t/ha of non-damaged tomato fruits when *T. absoluta* was managed using EPNs combined with neem, while a comparative yield of 9.7 t/ha was also obtained using the synthetic insecticide coragen® SC (20% Chlorantraniliprole).

Some plant extracts have been reported to have effect on plant growth, yield and fruit quality (Adebayo *et al*., 2007; Ahmed *et al*., 2009). When used to control *Alternaria solani* in tomato, plant extracts of jimson weed (*Datura stramonium*) and garlic (*Allium sativum*) resulted in yield increase of 76.2% and 66.7%, respectively, as compared to infected control (Nashwa and Abo-Elyousr, 2013). Plant extract from *T. vogelii* and *Petiveria alliacea* were used by Adebayo *et al*. (2007) to control insect pests on cowpea and increased the number of leaves and flowers per plants as compared to untreated control, but were not significantly different from deltamethrin 2.8 EC. The pod weight/plant, mean pod weight, number of seeds/pod, seed weight/pod, mean seed weight, and seed yield were also significantly higher in all the treated plots than untreated control.

Olaitan and Abiodun (2011) also used plant extracts from *T. vogelii and Petiveria alliacea* against field insect pests of cowpea and obtained that application of the extracts, irrespective of concentrations, significantly reduced pod damage and increased grain quality compared with untreated control. In a study to control bean pests using plant extracts, *T. vogelii* recorded higher yield, followed by *T. diversifolia*, *V. amygdalina*, positive control (lambda-cyhalothrin pyrethroid, Syngenta) and negative control, respectively (Mkenda *et al*., 2015).

In their study on mango, El-Sharony *et al*. (2015) obtained incresed number of new shoots, shoot length, shoot thickness, number of leaves per shoot, and leaf area by applying algae either alone or in combination with one or both water extracts of roselle (*Hibischus sabdariffa* L.) and garlic. These treatments also improved fruit set, yield and fruit quality (total soluble solids and ascorbic acid). Ahmed *et al.* (2009), reported increased yield (47% higher than control) of peach (*Prunus persica*) by application of garlic extract at a concentration of 4%. The same treatment also resulted in heavier and larger fruits with higher total soluble solids and lower titrable acidity as compared to other treatments.

The effects of entomopathogens and plant extracts on plant growth, yield and quality could be strain/species dependent (Elena *et al*., 2011; El-Sharony *et al*, 2015; Allegrucci *et al*., 2017). Therefore, it is important that any study using these biorational agents on a crop should also document their possible effects on these parameters.

2.7 Principles and practices of Integrated Pest Management

2.7.1 Brief history of IPM

The Integrated Pest Management (IPM) concept was first conceived by entomologists as a result of observed failures of synthetic pesticides to effectively control crop pests. These failures were mainly caused by pest resistance to used pesticides and pest outbreak induced by broad-spectrum pesticides that also killed natural enemies. The concept is now applied in all fields of crop production in addition to insect pests (Barzman *et al*., 2015).

Integrated pest management concept highlights that it not viable to rely on a single method to manage a particular pest even if that method is the most effective. This is because in long run the pest can develop surviving strategies to overcome that method and become less controllable as never before (IPPC, 2010). According to Barzman *et al*. (2015), IPM is not static and it is affected by several factors such as cropping practices, pest prevalence and pressure, state and presence of semi-natural habitats, research efforts, producers' behaviours and funds availability among others.

It has been observed that IPM can be successful if it effectively engages farmers in its implementation. Since IPM interventions cannot be generalised over all areas due to dissimilarities in ecological, geographical, social, and economic factors, famers are expected to be participatorily involved to deal with varied local conditions (Barzman *et al*., 2015).

The best approach has been to engage farmers and place the control of small-scale agroecosystems in the hands of these people who manage them (Braun and Duveskog, 2008). For instance, in Sri Lanka, farmers who were actively involved in IPM reduced pesticide applications from 3.8 to 1.5 times per season in rice farming and each of them could enumerate an average of four natural enemies as compared to 1.5 in control group (Trip *et al*., 2005). In Rwanda, farmers who were actively engaged in IPM were able to differentiate a pest from a beneficial organism by observing and analysising their behaviour on field; they were enabled to take decision on which action to take. This helped in reducing pesticide applications and enhanced activity of natural enemies. Among these farmers, 76% and 73% had increases in yield and income, respectively, by at least 50% (Somers *et al*., 2017).

2.7.2 Principles of integrated pest management

Eight principles were set in place to guide people applying IPM at different levels, namely researchers, crop production advisors, and producers. These principles help to understand how IPM concept can be applied and help achieving sustainable food production (Barzman *et al*., 2015; Pretty and Bharucha, 2015). They are effective when applied considering a wide scale of space and time instead of a separate crop or season (Barzman *et al*., 2015).

The first IPM principle emphasizes the necessity to prevent and or suppress pests through different ways such as use of resistant varieties and healthy planting materials, respect of crop rotation, adequate land preparation, proper fertilization and water management, conservation and boosting of natural enemies. Hygiene of farm tools and equipment to avoid dispersal of pests and diseases, respect of plant density, synchronised sowing, mulching, and pruning are also the recommended practices among others. The main aim of this principle is not to entirely eradicate the pest population, but to keep it below the level which inflicts significant damage (Barzman *et al*., 2015). Under this principle, healthy substrate and planting material are of particular importance (Van derWolf *et al*., 2013).

The second IPM principle stipulates that, whenever possible, appropriate methods and tools have to be used to monitor destructive pests. This can be achieved through field observations supplemented with knowledgeable quick detection, predicting and warning systems as well as regular consultation with experts in the field (Pretty and Bharucha, 2015). The third IPM principle states that thresholds obtained as a result of the reliable monitoring system, have to guide when to undertake any control strategy against a particular harmful organism.

The thresholds that were fixed for some destructive organisms have to be observed; while for other pests where the thresholds are not yet established, effort has to be made to set them (Barzman *et al*., 2015). Action should be taken whenever the pest population is at economic threshold (pest population density or extent of crop damage at which the value of crop destroyed exceeds the cost of controlling the pest) to avoid that it reaches economic injury level (lowest pest population density or extent of crop damage that can cause yield losses equal to the pest management cost) (Mantzoukas and Eliopoulos, 2020).

The fourth IPM principle underlines that alternative methods to synthetic pesticides have to be given priority as long as they provide effective pest control. Synthetic pesticides should be used as last resort when other methods have failed to keep the pest population below economic threshold (Tang *et al*., 2010; Barzman *et al*., 2015). According to the fifth IPM principle, the opted control strategies should be as selective as possible to the targeted organisms, without having side effects on natural enemies, humans, and other non-targeted organisms (Barzman *et al*., 2015). The sixth IPM principle states that attention has to be taken to use the necessary levels of the pesticide (application dosage and frequency) or other pest management options. According to this principle, farmers are urged to consider reducing the recommended levels, by monitoring the pest dynamics, in order to ensure that pesticide residues in the produce are as low as possible, and also to minimise the risks for pest resistance development (Tang *et al*., 2010; Barzman *et al*., 2015).

The seventh IPM principle elaborates that when a pest is known to possess ability to develop resistance against a given control option and when its control requires repeated pesticide application; strategies to prevent resistance development, such as use of various pesticides with different mode of actions, should be applied. According to the eighth IPM principle, evaluation should be carried out to assess the success or failure of used pest management options, considering monitoring and the pesticide use records, in order to take necessary action (Barzman *et al*., 2015).

2.7.3 Typology of IPM

Pretty and Bharucha (2015) discussed that IPM approaches can be grouped into four main types (Table 2.7) starting from the restrained or/and careful use of pesticides and ending with the habitat/ecosystem management.

Table 2.2 Typology of IPM approaches

No.	Type of IPM	Example of application
1a	Replacement of pesticide products with other products	Replacement of a highly toxic synthetic insecticide with a biorational pesticide like azadirachtin.
1b	Careful use of pesticides	Using selective pesticides, respect of action thresholds, and directed pesticide application to the targeted pest.
2	Breeding	Incorporation of resistance genes into an existing organism (case of *Bacillus thuringiensis* (Bt) maize).
3a	Release of living organisms to disturb or diminish the pest population	Natural enemies (parasitoids, predators, entomopathogens) can be released for pest control. Sterile males can be bred and released to compromise females' fertility, reducing pest population.
3b	Use of pheromones traps	Gummy and pheromone traps used to trap pests.
4	Habitat management	Crop rotation, mulching, raised beds in water-lodged conditions, use of push and pull plants, use of wild flowering plants to boost activity of natural enemies.

Source: Adapted from Pretty and Bharucha (2015)

One successful story of the first type of IPM, case of replacement of pesticide product(s) with others, is the use of neem-derived pesticides, which are successfully used in management of various pests (Giorgini *et al*, 2019) and are recommended to be used in IPM programmes depending on conservation or augmentation of natural enemies (Arnó and Gabarra, 2011). An example of careful use of pesticides is the case of Vietnam in rice fields where farmers supressed pesticide sprays in the first 40 days following transplanting, which boosted the activity of generalist predators and saved more than a half of the money that had been used, without compromising rice yield (Pretty and Bharucha, 2015).

The second type of IPM approaches, breeding, can also provide successful results in management of a particular crop pest. According to Lucas (2011), most of cultivated varieties are a result of selections, which might have focussed on yield improvement at the expense of pest/disease resistance traits. Thus, effort can be made to specifically breed for resistance

traits (Barzman *et al*., 2015). Some challenges associated with this IPM intervention are that breeders have to foresee the target pest/disease ahead of time and start breeding work well in advance to provide resistant variety when needed. Besides, pests/diseases can exhibit selection pressure to resistant traits when a resistant variety is used in a large area (Pretty and Bharucha, 2015). Furthermore, people have different perceptions on genetically transformed crops, which hinder the wide use of this quick breeding method (Wunderlich and Gatto, 2015).

For the third type of IPM, the widely know success story of deploying living organisms is *Epidinocarsis lopezi*, a parasitic wasp from Latin America which was released in Central and West Africa to control cassava mealybugs (*Phenacoccus manihoti*) (Pretty and Bharucha, 2015). In Bangladesh, use of pheromone traps against *Batrocera cucurbitae*, a fruit fly that was a big threat to cucurbits, increased yield from 40% to 130%, reduced the number of insecticide applications from 15 to 0, and increased income up to 300% (Rakshit *et al*., 2011).

One of the successful applications of the fourth type of IPM interventions, habitat management, is the control of cabbage aphids (*Brevicoryne brassicae*), diamondback moth (*Plutella xylostella*) and white butterfly (*Pieris rapae*) in China by conservation biological control. This resulted in reduction of pesticide use by 20% to 70% (Liu *et al*., 2014). The main purpose of this IPM approach type is to ensure diversity in time and space by several approaches such as domestication of wild plants and use them as habitat for beneficial organisms, or in crop rotation to break pest and/or disease cycle (Pretty and Bharucha, 2015).

2.7.4 Obstacles to IPM in developing countries

Parsa *et al*. (2014) conducted a survey where the respondents were the participants in a workshop under the topic 'IPM in Developing Countries", in November 2011. The following main obstacles were mentioned: limited assistance to farmers, unfavourable government policies and support, limited education levels of farmers, difficulties in practicing IPM as compared to conventional pest management, and influence of pesticide manufacturers. Other obstacles include, but are not limited to, limited funds, limited access to IPM inputs and knowledge, farmers' resistance to change, insufficiency of qualified IPM experts, collective actions required within farming community, limited market incentives, and shortage of IPM training programmes in universities and other training institutions. Thus, developing countries like Rwanda should take actions to address these obstacles to ensure effective IPM implementation and sustainable agriculture.

CHAPTER THREE

POTENTIAL OF ENTOMOPATHOGENIC NEMATODE ISOLATES FROM RWANDA TO CONTROL THE TOMATO LEAF MINER, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae)

Abstract

Tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), is an invasive pest that was detected for the first time in Rwanda in 2015. This study assessed the potential of using local isolates of entomopathogenic nematodes (EPNs) in the management of *T. absoluta* in Rwanda. Six EPNs including four locally isolated strains: *Steinernema* sp. RW14-M-C2a-3, *Steinernema* sp. RW14-M-C2b-1, *S. carpocapsae* RW14-G-R3a-2, and *Heterorhabditis bacteriophora* RW14-N-C4a, and two exotic species: *S. carpocapsae All* and *H. bacteriophora* H06 were evaluated. These two exotic EPN species were used as positive controls, while sterile tap water was used as negative control. Three bioassays were conducted in laboratory, using a tomato leaflet with third instar *T. absoluta* larva in gallery and 9-cm Petri-dishes as bioassay arenas in a completely randomized design with three replications. The EPNs were applied at a volume of 1 mL containing 500 infective juveniles per leaflet. Larval mortality was checked continuously for 96 h at 24 h intervals. The results revealed that all the tested EPNs were able to find and kill *T. absoluta* larvae inside the leaf galleries, and their efficacy increased with exposure time. The pathogenicity effects were significantly different ($p < 0.05$) among EPNs. In the first 24 h after inoculation, the efficacy of local EPN isolates (53.3% - 96.7%) was significantly higher than the one of exotic species (0.0% - 26.7%). The efficacy of three Rwandan EPN isolates, *Steinernema* sp. RW14-M-C2a-3, *Steinernema* sp. RW14-M-C2b-1 and *S. carpocapsae* RW14-G-R3a-2 was not significantly different from 24 to 96 h after inoculation, except for *S. carpocapsae* RW14-G-R3a-2 during 24 h after inoculation in bioassay three. There was a non-significant difference among all the EPN isolates after 96 h of exposure. The potential locally isolated EPNs against *T. absoluta* was investigated for the first time through this. Field experiments should be conducted to fully explore the possibilities of using local EPN isolates in integrated pest management of *T. absoluta* in Rwanda.

Keywords: *Tuta absoluta*, Entomopathogenic nematodes, *Heterorhabditis*, *Steinernema*, Local isolates, Biological control, Rwanda

3.1 Introduction

Tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), is an invasive pest originating from South America and was detected for the first time in Rwanda in the year 2015 (FAO, 2015). This pest is the major threat to tomato production as it can cause up to 100% yield loss under both greenhouse and open-field conditions (Biondi *et al*., 2018).

Chemical control remains the only option readily available in areas that are newly infested by *T. absoluta* (Brévault *et al*., 2014). However, the short developmental period of this pest and its many generations per year lead to numerous insecticide sprays in one season (Biondi *et al*. 2018). This facilitates the development of resistant pest strains to frequently used insecticides (Haddi *et al*., 2017) and leads to destruction of natural enemies (Macharia *et al*., 2009). The limited effectiveness in addition to the hazardous nature of chemical insecticides (Macharia *et al*., 2009), triggers the need for integrated pest management (IPM) and use of pest control actions that assure positive economic, ecological, and sociological effects (Blake *et al*., 2007).

Biological control is one of the safe ways of managing agricultural pests as it has no harmful effects on the environment and human health. Among biological control methods, entomopathogenic nematodes (EPNs) have the potential of being used effectively against *T. absoluta* (Van Damme *et al*., 2016; Biondi *et al*., 2018). The potential of EPNs was also evidenced against a diversity of other economically important pests (Wraight *et al*., 2017). However, no research had been conducted in Rwanda to explore the possibilities of including these EPNs in IPM of tomato crop.

Entomopathogenic nematodes belong predominantly to *Steinernematidae* and *Heterorhabditidae* families; they are obligate parasites that kill insects with the help of mutualistic bacteria (*Xenorhabdus* spp. and *Photorhabdus* spp., respectively) which live in gut/intestine of infective juveniles (IJs) (Stock, 2015). They can be effective against many soil-borne pests and others that live in galleries due to the conducive environment (protection against desiccation and ultraviolet light) for their IJs (Garcia-del-Pino *et al*., 2013). The IJs, which are the only free-living stage enter the host body via the natural openings or even through the soft body. Once inside the body, the bacteria cells released by IJs multiply quickly and kill the host in 24 to 72 h (Gözel and Kasap, 2015). Moreover, these bacterial cells digest host tissues and release antibiotics, which protect the killed host against saprophytes and scavengers, thus permitting the nematodes to develop and reproduce (Griffin *et al*., 2005). There might be one to three nematode generations depending on the size of the host.

When host nutrients are exhausted, the IJs sequester the bacteria in their intestines, leave the host and search for a new one; but when they miss a new host, the IJs can persist for months in moist soil (Stock, 2015).

It is recognized that the environment determines the success or failure of EPNs because of the possible differences in persistence, virulence, host range, and familiarity to habitats between local and non-local EPN isolates (Lacey and Georgis, 2012). The target host and the environment where EPNs will be applied should be considered when designing a control programme using EPNs. Thus, screening several nematode isolates against a particular target host in a specific environment is a prerequisite in development of any control programme using EPNs (Biondi *et al*., 2018). Four new EPN strains were isolated from semi-natural and small-holder farming habitats of Rwanda and maintained in Biological Control Laboratory – EPN Production Facility at Rwanda Agriculture and Animal Resources Development Board (RAB) (Yan *et al*., 2016). The objective of this study was to determine the potential of the EPNs against *T. absoluta* under laboratory conditions.

3.2 Materials and methods

The present study was carried out in the Biological Control Laboratory - EPN Production Facility of RAB (Holmes *et al*., 2015). The average annual rainfall and temperature of the locality are 1039 mm and 19^{o}C, respectively (Ndabamenye *et al*., 2013).

3.2.1 Source and mass production of EPNs

Six EPNs including four local isolates and two exotic species, maintained in the Biological Control Laboratory - EPN Production Facility of RAB were used for the study. The local EPNs were *Steinernema* sp. RW14-M-C2a-3, *Steinernema* sp. RW14-M-C2b-1, *S. carpocapsae* RW14-G-R3a-2, and *Heterorhabditis bacteriophora* RW14-N-C4a, which were isolated from semi-natural and small-holder farming habitats of Rwanda in the year 2014 (Yan *et al*., 2016). The two exotic EPN species, *S. carpocapsae* All and *H. bacteriophora* H06 were obtained from Lvbenyan Biotech Ltd., Guangdong Institute of Applied Biological Resources (GIABR) in China (Kajuga *et al*., 2018). The exotic species were used as standard checks as they are among the most used to control foliar and soil insect pests (Lacey and Georgis, 2012). *In-vivo* method of EPN mass production was followed in the aforementioned laboratory, using last instar larvae of *Galleria mellonella* L. (Lepidoptera: Pyralidae), which is mostly used for this purpose as it is easily infested by nematodes (Kaya and Stock, 1997).

The *G. mellonella* larvae, killed by EPNs, were moved to a white trap (White, 1927) for the infective juveniles (IJs) to come out of the cadavers. IJs were harvested, rinsed in distilled water, and stored at 7^{o}C for less than one week. Since EPNs do not go through the complete dormancy stage and continue to consume their limited energy during storage (Mahmoud, 2016), they were used in bioassays when still fresh (in less than seven days) after harvesting from the white trap. The EPNs were allowed to acclimatise at room temperature for one hour before their use, and their viability was checked under a stereomicroscope with × 60 magnification, where live IJs were moving actively (Garcia-Del-Pino *et al*., 2013). These EPNs were used in bioassay once more than 90% of IJs were viable (Kajuga *et al*., 2018).

3.2.2 Source of *Tuta absoluta* larvae

To secure the source of laboratory specimens (tomato leaflets with *T. absoluta* larvae in galleries), a field of tomato, *Solanum lycopersicum* cv. Roma, was established in Bugesera District, Rweru Sector. This area was selected because it is the hot spot of *T. absoluta* infestation in Rwanda. Tomato crop was established in November 2018 (two months before starting bioassays) following the package recommended for field cultivation of the crop in Rwanda. Insecticides were not applied from two weeks after planting to favour the quick development of *T. absoluta* in the field. Tomato leaflets containing *T. absoluta* larvae inside the galleries were harvested from this naturally infested tomato field with caution to have only one larva per leaflet. These leaflets were transported in a cloth bag to the laboratory and used in bioassays in less than 12 h after their collection.

3.2.3 Pathogenicity test

Three bioassays were conducted from January to February 2019. Each bioassay was carried out as a full experiment in a completely randomized design with three replications. Ten Petri-dishes of 9-cm diameter each, lined with three moistened filter paper discs, were used as bioassay arenas per each treatment in a replication. Tomato leaflets containing third instar *T. absoluta* larvae (3.85 -5.65 mm) in galleries were carefully selected for use in the bioassays. Each Petri-dish received only one leaflet. An hour before each bioassay, nematode concentrations were calculated as per Navon and Ascher (2000) and adjusted to the required concentration of 500 IJs/mL (Batalla-Carrera *et al*., 2010; Mutegi *et al*., 2017) using sterile tap water. Thereafter, 1 mL of water containing 500 IJs of EPNs was applied on both sides of each leaflet, using a sterilised pipette for each EPN isolate (Batalla-Carrera *et al*., 2010) and

sterile tap water was used as control. The Petri-dishes were sealed by parafilm to protect them against dehydration and prevent the escape of the larvae. The Petri-dishes were then maintained at a temperature of 25°C ± 2°C in dark. Fresh leaflets were added after two days to prevent starvation of larvae.

3.2.4 Data collection and analysis

Petri-dishes were opened 24, 48, 72, and 96 h after inoculation to check the status of larvae whether dead or alive. Dead larvae were recognised by being unable to respond to stereoscopic light or to probing using the tip of camel's hair brush (Van Damme *et al*., 2016). Dead and alive larvae at the end of bioassay were dissected under the stereomicroscope to determine the presence or absence of nematodes in their bodies (Kajuga *et al*., 2018). The number of dead larvae at each observation period was used to compute the observed mortality (%), which was obtained by dividing the number of dead larvae per treatment by the total number of larvae per treatment and multiplying the result by hundred. When there was mortality in Petri-dishes treated with water, this was considered as natural mortality and was used to correct the mortality observed in Petri-dishes where EPNs were applied. This correction was done using Schneider-Orelli's formula (Püntener, 1981) as follows:

$$\text{Corrected mortality} = \frac{\text{Observed mortality (\%)} - \text{Control mortality (\%)}}{100 - \text{Control mortality (100)}} \times 100.$$

Data were checked for normality before subjecting them to statistical analysis. Data on corrected mortality at 48 and 72 h after inoculation were arcsine-transformed as per Rangaswamy (2013). The general linear model (GLM) was used to determine whether the effect of EPNs on *T. absoluta* larvae mortality was significantly different or not. Tukey's honestly significant difference (HSD) test was used to separate the means. These analyses were performed, using the Statistical Analysis System package, SAS software version 9.2 (SAS Institute, 2010), and the level of significance was fixed at 5%.

The statistical model fitted for this experiment was: $Y_{ij} = \mu + t_i + e_{ij}$
Where Y_{ij} = larva mortality, μ = overall mean, t_i = effect of i^{th} treatment of entomopathogenic nematode (i = 1,2,3,4,5,6) and e_{ij} = error term.

3.3 Results

All EPN isolates evaluated were able to find, infect, and kill *T. absoluta* larvae inside the tomato leaf galleries, however, with different levels of pathogenicity (Table 3.1).

Table 3.1 Summary output of GLM analysis for *T. absoluta* larvae mortality after different time following EPNs application at 500 IJs/mL

Bioassay	Sources of variation	df	F	P	Significance
One	EPNs at 24 h of exposure	5, 12	48.29	< 0.0001	***
	EPNs at 48 h of exposure	5, 12	9.95	0.0006	***
	EPNs at 72 h of exposure	5, 12	71.06	< 0.0001	***
	EPNs at 96 h of exposure	5, 12	1.00	0.4582	Ns
	Exposure time	3,48	95.49	< 0.0001	***
	EPNs x Exposure time	15,48	7.36	< 0.0001	***
Two	EPNs at 24 h of exposure	5, 12	27.46	< 0.0001	***
	EPNs at 48 h of exposure	5, 12	423.82	< 0.0001	***
	EPNs at 72 h of exposure	5, 12	3.09	0.0508	Ns
	EPNs at 96 h of exposure	5, 12	-	-	Ns
	Exposure time	3,48	95.04	< 0.0001	***
	EPNs x Exposure time	15,48	12.86	< 0.0001	***
Three	EPNs at 24 h of exposure	5, 12	204.85	< 0.0001	***
	EPNs at 48 h of exposure	5, 12	32.69	< 0.0001	***
	EPNs at 72 h of exposure	5, 12	4.82	0.0119	*
	EPNs at 96 h of exposure	5, 12	-	-	Ns
	Exposure time	3,48	153.75	< 0.0001	***
	EPNs x Exposure time	15,48	19.28	< 0.0001	***

* and *** shows significance at $p < 0.05$ and $p < 0.001$ respectively; Ns indicates non-significance; the sign – indicates where GLM was not possible because there was no variation among treatments. Each of the three bioassays represented a full experiment with three replications.

The recorded mortality differed significantly among the tested EPNs. However, there was a non-significant difference among EPNs ($p > 0.05$) after 72 h of exposure for bioassay two and after 96 h of exposure for all bioassays. When data (arcsine-transformed) were analysed considering exposure time and EPNs x exposure time combination as treatments, it was observed that both affected significantly ($p < 0.001$) *T. absoluta* larval mortality (Table 3.1). In the first 24 h after inoculation, the efficacy of all local EPN isolates was significantly higher than the two exotic EPN species in the three bioassays ($p < 0.001$), except for *H. bacteriophora* RW14-N-C4a in bioassay two (Table 3.2).

Table 3.2 Mortality of *Tuta absoluta* larvae (mean ± SD) in leaf galleries treated with local and exotics EPNs using the concentration of 500 IJs/mL

EPN isolates	*Tuta absoluta* larval mortality (%)			
	24 h	**48 h**	**72 h**	**96 h**
Bioassay One				
St. sp. RW14-M-C2a-3	80.0 ± 10.0 a	96.7 ± 5.8 a	100 ± 0.0 a	100 ± 0.0 a
St. sp. RW14-M-C2b-1	83.3 ± 15.3 a	92.5 ± 6.6 a	100 ± 0.0 a	100 ± 0.0 a
St. carp. RW14-G-R3a-2	90.7 ± 5.8 a	95.8 ± 7.2 a	100 ± 0.0 a	100 ± 0.0 a
Het. bact. RW14-N-C4a	53.3 ± 5.8 b	96.7 ± 5.8 a	100 ± 0.0 a	100 ± 0.0 a
St. carp. ALL	6.7 ± 5.8 c	40.0 ± 10.0 b	84.7 ± 6.1 b	95.8 ± 7.2 a
Het. bact. H06	26.7± 5.8 c	85.8 ± 5.2 a	100 ± 0.0 a	100 ± 0.0 a
CV	15.26	13.21	2.21	2.97
Bioassay Two				
St. sp. RW14-M-C2a-3	83.3 ± 20.8 ab	100 ± 0.0 a	100 ± 0.0 a	100 ± 0.0 a
St. sp. RW14-M-C2b-1	96.7 ± 5.8 a	100 ± 0.0 a	100 ± 0.0 a	100 ± 0.0 a
St. carp. RW14-G-R3a-2	90.0 ± 10.8 a	100 ± 0.0 a	100 ± 0.0 a	100 ± 0.0 a
Het. bact. RW14-N-C4a	53.3 ± 11.5 bc	100 ± 0.0 a	100 ± 0.0 a	100 ± 0.0 a
St. carp. ALL	3.3 ± 5.8 d	35.9 ± 7.6 b	88.4 ± 11.5 a	100 ± 0.0 a
Het. bact. H06	23.3 ± 15.3 dc	39.2 ± 5.6 b	92.6 ± 6.4 a	100 ± 0.0 a
CV	21.76	3.13	8.82	-
Bioassay Three				
St. sp. RW14-M-C2a-3	96.7 ± 5.8 a	100 ± 0.0 a	100 ± 0.0 a	100 ± 0.0 a
St. sp. RW14-M-C2b-1	96.7 ± 5.8 a	100 ± 0.0 a	100 ± 0.0 a	100 ± 0.0 a
St. carp. RW14-G-R3a-2	70.0 ± 0.0 b	96.3 ± 10.9 ab	100 ± 0.0 a	100 ± 0.0 a
Het. bact. RW14-N-C4a	46.7 ± 5.8 c	85.6 ± 6.8 b	96.3 ± 6.4 ab	100 ± 0.0 a
St. carp. ALL	0.0 ± 0.0 e	17.4 ±10.9 c	84.7 ± 6.1 b	100 ± 0.0 a
Het. bact. H06	26.7 ± 5.8 d	92.6 ± 6.4 ab	95.8 ± 7.2 ab	100 ± 0.0 a
CV	8.40	7.82	4.84	-

St = *Steinernema*; *Het = Heterorhaditis*; *St. carp. = Steinernema carpocapsae; Het. bact. = Heterorhabditis bacteriophora;* Means followed by different letters in the same column within the same bioassay are significantly different according to Tukey's test ($p \leq 0.05$). Each of the three bioassays represented a full experiment with three replications.

At 24 h, the entomopathogenic nematodes belonging to the *Steinernema* genus recorded higher *T. absoluta* larval mortality than the ones belonging to *Heterorhabditis.* At 48 h after inoculation, the efficacy of *H. bacteriophora* H06 was not significantly different from the one of local EPN isolates in bioassays one and three; but this lasted 72 h in bioassay two. In 72 h post-inoculation, all local EPN isolates had achieved 100% mortality in all bioassays, except *H. bacteriophora* RW14-N-C4a, which had 96.3% in bioassay three. The maximum mortality was achieved on different times of exposure to EPNs, ranging from 48 to 96 h (Table 3.2). Among the local (Rwandan) EPN isolates, *Steinernema* sp. RW14-M-C2a-3, *Steinernema* sp. RW14-M-C2b-1 and *S. carpocapsae* RW14-G-R3a-2, remained not significantly different in efficacy from 24 h to 96 h after inoculation, except that *S. carpocapsae* RW14-G-R3a-2 was significantly different from them only during 24 h after inoculation in bioassay three. *H. bacteriophora* RW14-N-C4a joined their group 48 h after inoculation for bioassays one and two, and 72 h for bioassay three; which revealed that it was not as effective. (Table 3.2).

3.4 Discussion

The ability of EPNs to reach and kill *T. absoluta* larvae in leaf galleries was also reported by Batalla-Carrera *et al*. (2010), Van Damme *et al*. (2016), and Kamali *et al*. (2018). The local EPN isolates were able to kill *T. absoluta* and had even been found to be effective against white grubs in Rwanda (Kajuga *et al*., 2018), while it was not easy to find EPNs which can kill them (Laznik and Trdan, 2015). Although EPNs live naturally in soil, different researchers found that they can be used on above-ground parts of the plant to control effectively the pests living in cryptic habitats like in leaf galleries (Batalla-Carrera *et al*., 2010; Garcia-del-Pino *et al*., 2013); which concurs with the results of the present study under laboratory conditions.

In the present study, the third instar larvae were used; other studies revealed that EPNs were able to find and kill all the four larval instars inside or outside the leaf galleries (Batalla-Carrera *et al*., 2010; Van Damme *et al*., 2016). Batalla-Carrera *et al*. (2010) found that *T. absoluta* larval stage was the most vulnerable to EPNs. They thus emphasized the necessity to apply EPNs on the above-ground part of the tomato plant to ensure effective control of this pest using the most suitable isolates against a particular pest in a given environment.

Different pathogenicity levels displayed by the studied EPNs agree with other studies, using different EPN isolates (Gözel and Kasap, 2015; Van Damme *et al*., 2016). This underlines the necessity of EPNs screening and selection as emphasized by Sharma *et al*. (2011), Biondi *et*

al. (2018) and Saleh *et al*. (2020) in a view to boost their efficacy. The higher efficacy of local EPN isolates than the exotics could be explained by the fact that these EPNs were isolated in Rwanda (Yan *et al*., 2016), and they might be more adapted to the local conditions than the exotics, which were isolated in a completely different environment. These results agree with the earlier findings where locally isolated biological control agents, including EPNs, performed better than exotics (Lima *et al*., 2017).

Higher pathogenicity of EPN isolates belonging to *Steinernema* genus than *Heterorhabditis* had also been reported by Batalla-Carrella *et al*. (2010) who obtained 76.3% mortality of *T. absoluta* larvae inside the leaf galleries using *H. bacteriophora*, while it was 88.6% and 92.0%, using *S. carpocapsae* and *S. feltiae*, respectively, at a dosage of 60 IJs/cm^2. Furthermore, *Steinernema* genus was observed to be the most virulent among different EPN species by different other researchers (Van Damme *et al*., 2016; Mutegi *et al*., 2017; Kamali *et al*., 2018), which is consistent with the obtained findings.

Higher efficacy of EPNs belonging to the *Steinernema* genus could be due to bacteria associated with their genus, and ambusher strategy for host scavenging with standing and jumping behaviours, which helps them to attach on the host (Lacey and Georgis, 2012; Stock, 2015). Furthermore, it was reported that some species of *Steinernema* genus possess both cruiser and ambusher strategies; which make them more efficient in finding their host. The stand and jump behaviours, as well as this intermediate foraging strategy, have not been reported in the *Heterorhabditis* genus (Lewis *et al*., 2006). However, because of their dorsal tooth EPNs belonging to the *Heterorhabditis* genus would be expected to penetrate directly the insect body through the thin wall area between the segments (Griffin *et al*., 2005), and they would be more pathogenic than *Steinernema* genus. However, this thought was not evidenced by the results of the present study.

It is established that after EPNs have entered the host, the bacteria cells released by the IJs multiply quickly and kill the host in 24 - 72 h after infection (Gözel and Kasap, 2015; Van Damme *et al*., 2016; Jaffuel *et al*., 2020). This was verified in the present study where all local EPN isolates caused between 53.3% and 96.7% mortality just within the first 24 h after inoculation, while in 72 h, they all had caused between 96.3% and 100% mortality.

The quick kill behaviour of EPNs is beneficial for foliar applications where it can be guaranteed that before EPNs are killed by adverse environmental conditions such as desiccation and ultraviolet light, they would have searched, found and invaded their hosts in leaf galleries. Kim *et al*. (2006) reported that EPNs were able to survive 12 h after foliar spray

on Chinese cabbage. This gives hope that the application of EPNs on the aboveground part of the plant would yield good results because EPNs will have at least 12 h to find and invade the host no matter whether it is on the leaf surface or inside the leaf gallery where the more convenient environment is guaranteed for survival.

During this study, one fixed dosage of 500 IJs/mL was used because the main purpose was to screen the local EPN isolates and find out the most effective ones against *T. absoluta* for further investigations. Other researchers found that the higher the dosage, the higher the efficacy. For instance, Youssef (2015) observed that with 3 dosages of 250, 500, and 1000 IJs/ mL for *S. carpocapsae*, mortality rates of *T. absoluta* larvae reached to 80%, 100%, and 100%, respectively. A similar trend was obtained by Batalla-Carrera *et al*. (2010), Mutegi *et al*. (2017), Yuksel *et al*. (2018), and Kajuga *et al*. (2018) on various pests. This could be because a high number of EPNs would result in a high number of symbiotic bacteria released in the host's body and thus enhanced killing speed owing to increase digestion of host tissues by toxins and hydrolytic enzymes secreted by these bacteria (Van Damme *et al*., 2016).

The observed efficacy and rapid action of EPNs make them able to compete with conventional insecticides that are preferred due to their quick action among others (Macharia *et al*., 2009; Biondi *et al*. 2018). The safety, high virulence, ability to actively search for their hosts, mass production possibility, and compatibility with many pesticides are the other traits, which make EPNs a good option in IPM and potential substitutes for synthetic insecticides (Lima *et al*., 2017). Thus, further investigations should be carried out to determine their effectiveness under field conditions.

3.5 Conclusion

The results of this study revealed that local EPN isolates were able to find and kill *T. absoluta* larvae inside the leaf galleries under laboratory conditions and their efficacy increased with exposure time. The efficacy of local EPN isolates was significantly superior to that of the exotic species. This is the first study carried out in Rwanda to determine the potential of locally isolated EPNs against *T. absoluta*. The results of this study form the basis for further research. High EPN efficacy obtained under laboratory conditions cannot easily be extrapolated to field efficacy. Therefore, field experiments on tomato crop are justified to fully determine the potential of local EPN isolates against *T. absoluta* in Rwandan conditions.

CHAPTER FOUR

PATHOGENICITY OF SOME COMMERCIAL FORMULATIONS OF ENTOMOPATHOGENIC FUNGI ON THE TOMATO LEAF MINER, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae)

Abstract

The tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), is a major threat to tomato production in Rwanda. Laboratory bioassays were conducted to evaluate some commercial formulations of entomopathogenic fungi (EPFs) on *T. absoluta* larvae. The larvae, inside the leaf galleries, were obtained from the established tomato field. Commercial EPFs: Metatech® WP [*Metarhizium anisopliae* (Metschn.) Sorok, Strain FCM Ar 23B3], Beauvitech® WP [*Beauveria bassiana* (Bals.) Vuill., Strain J25], and Botanigard ES [*B. bassiana* (Bals.), Strain GHA] were tested in Petri-dishes against *T. absoluta* larvae at a concentration of 10^8 spores/mL. A synthetic insecticide, imidacloprid (Confidor SL 200) was included for comparison as a positive control, while water was used as a negative control. All the tested commercial EPF formulations were pathogenic to *T. absoluta* larvae in all conducted bioassays. Mortality rates increased with an increase in time (days). However, non-significant difference was observed in mortality of *T. absoluta* larvae treated with the commercial EPFs during the first three days in all bioassays. Highly significant differences ($p < 0.01$) in pathogenicity among treatments were observed from the fourth to sixth days after inoculation. Metatech® WP and Beauvitech® WP recorded the highest mortality rates (82.8% and 60.8%) with the LT_{50} values of 3.9 and 5.2 days, respectively, while imidacloprid caused the least larval mortality. Since the EPFs demonstrated high virulence level against the target pest, Metatech® WP and Beauvitech® WP should be advanced to field evaluation to determine their potential as alternatives to synthetic insecticides.

Keywords: *Tuta absoluta*, Entomopathogenic fungi, *Beauveria bassiana*, *Metarhizium anisopliae*, Biological control, Rwanda

4.1 Introduction

Despite its economic and nutritional importance, tomato production in Rwanda is challenged by various factors, including prevalence of pests and diseases, limited skills in pest management, and lack of appropriate pest management options (Clay and Turatsinze, 2014).

The tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), is a major threat to tomato production in Rwanda since 2015 (FAO, 2015). It damages the plant by mining the leaves and boring into the stems and fruits, resulting in reduced tomato yield and fruit quality (Brévault *et al*., 2014). So far, little has been done to develop integrated pest management (IPM) programme fitting Rwandan conditions for this pest. *Tuta absoluta* control in Rwanda is mainly based on synthetic insecticides, which affect the populations of beneficial organisms, especially pollinators and natural enemies, and cause water pollution and disturbance of aquatic ecosystems as well as human health problems (Shalaby *et al*., 2013). Furthermore, the continuous use of synthetic pesticides has been reported to result in the build-up of resistant biotype populations of *T. absoluta* (Yalçin *et al*., 2015).

Numerous studies have been conducted on entomopathogenic fungi (EPFs) to control a diversity of pests that showed to be highly effective (Contreras *et al*., 2014). Specifically, *Beauveria bassiana* (Bals.) Vuill. (Ascomycota: Hypocreales) and *Metarhizium anisopliae* (Metsch.) Sorok (Ascomycota: Hypocreales) have been used against insect pests for more than 120 years (Contreras *et al*., 2014; Nishi *et al*., 2020). These fungi have important features that make them the potential agents for biological control; they are widely distributed in nature, easily isolated from insect cadavers or from soil, can be cultured in laboratory on simple media and conserved by storing conidia in glycerol solutions, and easily mass-produced and formulated as bio-insecticides (Qazzaz *et al*., 2015.

Different scientists reported that *T. absoluta* control can be achieved in environmentally friendly way using *B. bassiana* (Qazzaz *et al*., 2015; El-Kichaoui *et al*., 2016) and *M. anisopliae* (Shalaby *et al*., 2013; Contreras *et al*., 2014). In laboratory experiments, these EPFs were observed to infect and control *T. absoluta* larvae inside the leaf galleries and the adults emerged from treated pupae (Youssef, 2015; El-Ghany *et al*., 2016). Since EPF mass production is cost-effective (Mantzoukas and Eliopoulos, 2020), they have substantial advantages of being used in biological control of *T. absoluta*.

In Rwanda, there are no fungal strains registered for *T. absoluta* control. Besides, the global insecticide resistance action committee (IRAC, 2011) recommended that the evaluation of the efficacy of different pesticides against *T. absoluta* in local conditions should be emphasized when designing an effective IPM programme. This study was carried out to determine the pathogenicity of three selected commercial formulations of EPFs, based on *B. bassiana* and *M. anisopliae*, against the Rwandan population of *T. absoluta* under laboratory conditions.

4.2 Materials and methods

4.2.1 Study site

The study was conducted at the Biological Control Laboratory, Rwanda Agriculture and Animal Resources Development Board (RAB).

4.2.2 Entomopathogenic fungi

Three commercial biopesticides based on EPFs were used in the study (Table 4.1). The EPFs were cultured on Potato Dextrose Agar (PDA) media and incubated at 25°C ± 1°C for seven days to confirm their viability before being used (Youssef, 2015). Observation under a stereomicroscope at × 40 magnification revealed that over 95% of spores had germinated in all the tested EPFs. Dilutions were carried out for each EPF product to achieve a concentration of 10^8 spores/mL.

Table 4.1 Commercial entomopathogenic fungi used in the experiment

Commercial name	Active ingredient and concentration	Manufacturer	Distributor
Metatech® WP*	*Metarhizium anisopliae* (Metsch.) Sorok, Strain FCM Ar 23B3, 5 x 10^9 CFUs/g	Dudutech Division, Flamingo Horticulture (K) Ltd, Naivasha, Kenya	Elgon Kenya Ltd.
Beauvitech® WP	*Beauveria bassiana* (Bals.) Vuill., Strain J25, 1 x 10^{10} CFUs/g	Dudutech Division, Flamingo Horticulture (K) Ltd, Naivasha, Kenya	Elgon Kenya Ltd.
Botanigard® ES	*Beauveria bassiana* (Bals.) Strain GHA, 2 x 10^{13} viable spores/1.14 L	LAM International Corp, USA 117 South Parkmont, Butte, MT59701.	Amiran Kenya Ltd

*WP = Wettable Powder, ES = Emulsifiable Suspension, FCM = False Codling Moth, CFU = Colony Forming Unit

Source: Products' labels

4.2.3 *Tuta absoluta* larvae

Leaflets infested with *T. absoluta* larvae in the galleries were collected from a tomato crop cultivated in September 2018 in a field located in Bugesera District, Rweru Sector. The tomato cultivar Roma was chosen because it is the most commonly grown by Rwandan farmers in open field conditions. The collected leaflets were transported in cloth bags to the laboratory and were kept for a maximum of 12 h before being used in the experiment.

4.2.4 Laboratory bioassays

Three successive bioassays were conducted on different dates from November through December 2018. Petri-dishes (9 cm diameter), lined with three moistened filter paper discs, were used as bioassay arenas. Each leaflet, with one third-instar larva (3.85 - 5.65 mm) of *T. absoluta* in gallery, was placed in a Petri-dish and treated on both sides with 2 mL of a respective EPF at the dosage of 10^8 spores/mL (Youssef, 2015), using a sterile pipette. The excess of the applied volume was drained on a filter paper. A synthetic insecticide, imidacloprid (Confidor SL 200) (1 mL/L), and sterilised tap water were used as positive and negative controls, respectively.

The Petri-dishes (n = 10) were supplied by moisture (1 mL water) as needed to avoid desiccation of leaflets and ensure continuous and adequate moisture for spore germination (Shalaby *et al*., 2013), while a fresh leaflet was added every other day to prevent starvation of *T. absoluta* larvae. The Petri-dishes were sealed by a parafilm membrane to prevent dehydration and the escape of the larvae and were maintained at 25°C ± 2°C in dark. Three bioassays where conducted and each was carried out as a completely randomized design with four replications.

4.2.5 Data collection and analysis

Larval mortality was evaluated daily for six days post-inoculation (Youssef, 2015). The dead larvae were moved to Petri-dishes lined with moistened filter paper to permit fungal growth on their bodies. After 5 - 10 days, the cause of death could be confirmed through fungal outgrowth on the dead larvae. The corrected mortality was calculated using Schneider-Orelli's formula (Püntener, 1981).

Data on corrected mortality were checked for normality; to obtain a normally distributed data set, different transformations like log, square-root, and arcsine transformations were tried and the best one chosen by interpreting the output (Rangaswamy, 2013). For the data of the first bioassay, square-root transformation was used for day four, while log-transformation was used for days five and six. For the data of the second and third bioassays, log transformation was used for days five and six, while the data on day four were analysed without transformation. The effect of treatments on *T. absoluta* larval mortality was evaluated, using the general linear model procedure. Means for statistically different treatments were separated, using Tukey's honestly significant difference (HSD) test at 5% level of significance. Lethal time to kill 50% of treated larvae (LT_{50}) was computed for all evaluated EPF formulations through probit analysis (Throne *et al*., 1995). Reciprocal square-root transformation of probit data was carried out before subjecting them to analysis of variance. All the analysis was performed using the Statistical Analysis System package SAS software version 9.2 (SAS Institute, 2010).

The statistical model fitted for this experiment was: $Y_{ij} = \mu + t_i + e_{ij}$
Where Y_{ij} = larva mortality, μ = overall mean, t_i = effect of i^{th} treatment of entomopathogenic fungi or control (i = 1, 2, 3, 4) and e_{ij} = error term.

4.3 Results

The entomopathogenic fungi (EPFs) used in this study, at a dosage of 10^8 spores/mL, were pathogenic to *T. absoluta* larvae in all conducted bioassays. In all treatments, mortality rates increased with the increase in time (days). They were very low in the first three days and started to be significantly different among the treatments ($p < 0.01$) from the fourth day after application. The formulation based on *M. anisopliae* (Metatech® WP) was not significantly different from *B. bassiana* (Beauvitech® WP) in all bioassays on days five and six, except on day five of bioassay one. In most cases, Metatech® WP recorded higher mortality rates than Botanigard®ES (*B. bassiana*, Strain GHA) and imidacloprid in all bioassays. The highest mortality rates observed were 82.8%, 60.8%, 48.8%, and 33.5% for Metatech® WP, Beauvitech® WP, Botanigard® ES, and imidacloprid (control), respectively. In all bioassays, the mortality observed in tomato leaflets treated with imidacloprid, the synthetic insecticide mostly used by farmers, was the least than EPF applications (Figure 4.1).

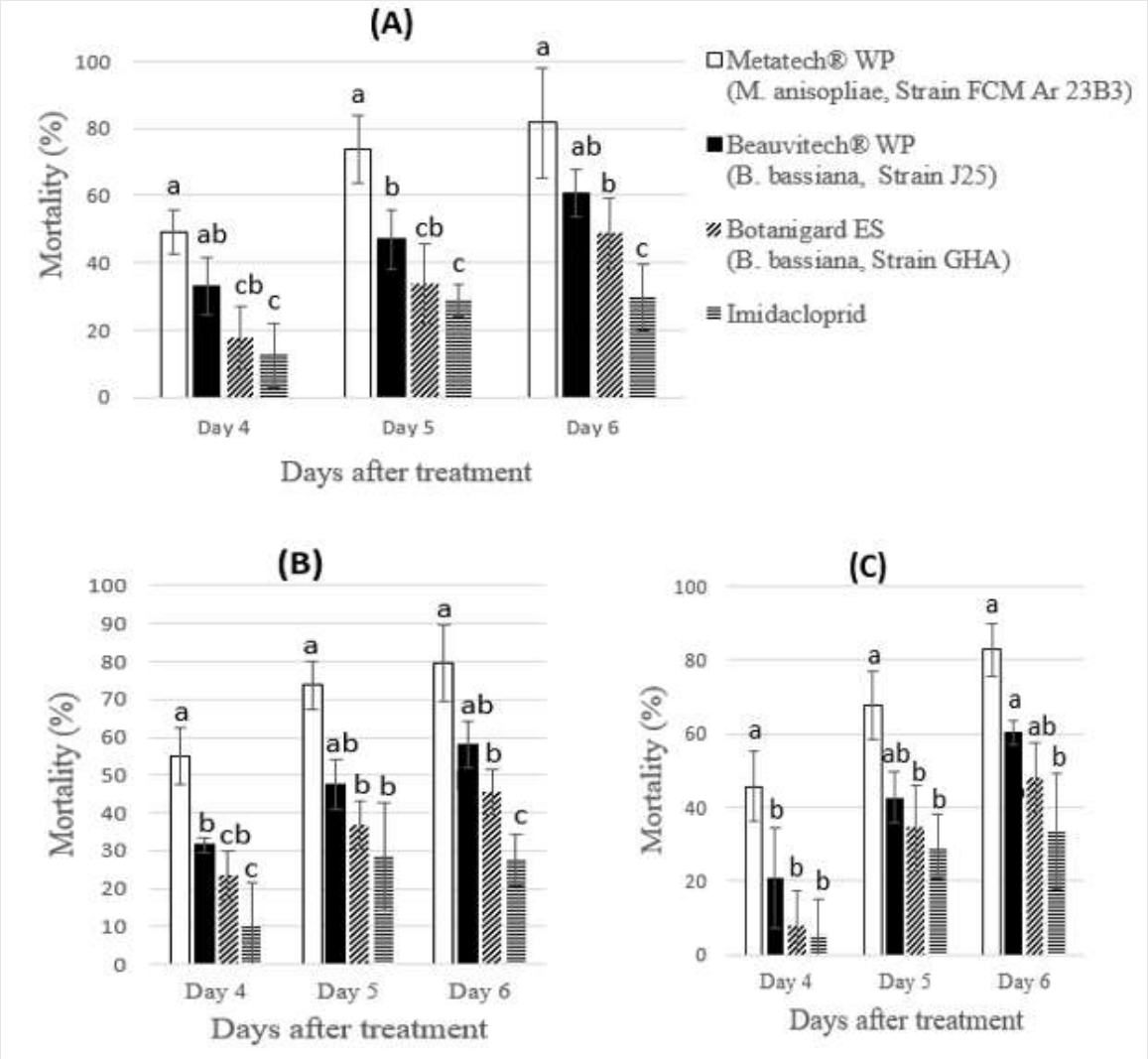

Figure 4.1 Mortality (%) of *Tuta absoluta* larvae treated with commercial EPF formulations (10^8 spores/mL) and imidacloprid (control) in bioassays one (A), two (B) and three (C). Different letters above bars (Mean ± SD) within the same day indicate significant difference according to Tukey test ($p \leq 0.05$). Each of the three bioassays represented a full experiment with four replications.

In all three bioassays, the lowest LT_{50} values were recorded by Metatech® WP (3.5 – 4.2 days), followed by Beauvitech® WP (5.2 – 5.3 days), Botanigard® ES (6.3 – 6.8 days), and imidacloprid (14.1 – 16.1), respectively (Figure 4.2). The pooled means of LT_{50} values were 3.9, 5.2, 6.6, and 14.9 days for Metatech® WP, Beauvitech® WP, Botanigard® ES, and imidacloprid, respectively.

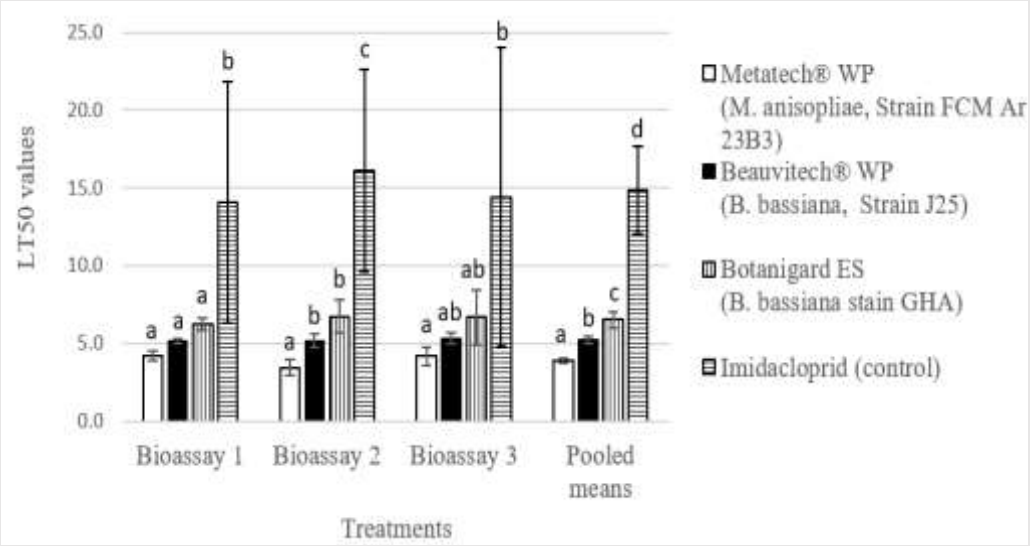

Figure 4.2 Lethal time in days to control 50% of *Tuta absoluta* larvae treated with commercial EPF formulations (10^8 spores/mL) and imidacloprid (control). Different letters above the bars (Mean ± SD) indicate significant difference within the same bioassay according to Tukey test ($p \leq 0.05$). Each of the three bioassays represented a full experiment with four replications.

4.4 Discussion

Various studies also reported that *T. absoluta* control could be achieved by using *M. anisopliae* (Shalaby *et al*., 2013; Contreras *et al*., 2014; Shiberu and Getu, 2017) and *B. bassiana* (Qazzaz *et al*., 2015; Youssef, 2015; El-Kichaoui *et al*., 2016). The results obtained form the basis for further studies on these EPFs in a view to find the appropriate ways of using them under field conditions. Higher virulence of *M. anisopliae* compared to *B. bassiana* was also reported by Murerwa *et al*. (2014) against the aphids *Rhopalosiphum padi* and *Metopolophium dirhodum*. Conflicting results were obtained by Moawad *et al*. (2017), who reported that *B. bassiana* was more effective than *M. anisopliae* in all treated larval instars of *Stomphastis thraustica* (Meyrick) (Lepidoptera: Gracillariidae), a leaf miner of *Jatropha curcas*. Similarly, Youssef (2015) observed a high mortality rate of *T. absoluta* larvae inside the galleries with *B. bassiana* (86.7%) than with *M. anisopliae* (76.7%), using a dosage of 10^8 spores/mL. This could be explained by the fact that the pathogenicity of a particular entomopathogen depends on strain/isolate and environment, among others (Borisade and Magan, 2014). Thus, screening different EPF species and strains against a particular target host is crucial in the development of any control programme (Georgis *et al*., 2006).

At the dosage of 10^8 spores/mL, the highest mortality rates recorded in the present study were 82.8% and 60.8% for *M. anisopliae* and *B. bassiana*, respectively. Other studies could obtain higher mortality levels of *T. absoluta* larvae with higher dosages. For instance, Youssef (2015) observed a mortality rate of 90% in *T. absoluta* larvae inside the galleries with *B. bassiana* at a dosage of 10^{10} spores/mL; while El-Kichaoui *et al*. (2016) obtained up to 95% mortality of *T. absoluta* larvae, using *B. bassiana* at a dosage of 2.5×10^7 spores/mL. This concurs with the findings of Shalaby *et al*. (2013) who reported a linear relationship between the mortality rate of *T. absoluta* and concentrations of *B. bassiana* and *M. anisopliae*.

The limited efficicacy of imidacloprid obtained in this study could be due to the ability of *T. absoluta* to develop resistant strains to the frequently used synthetic insecticides (Yalçin *et al*., 2015; Biondi *et al*., 2018). Likewise, the resistance of *T. absoluta* to indoxacarb and chlorantraniliprole was detected by Roditakis *et al*. (2013) in three laboratories belonging to the three different countries, Greece, Italy, and Spain. In Turkey, the resistance of *T. absoluta* to five commonly used insecticides, spinosad, indoxacarb, metaflumizone, and chlorantraniliprole, was also recorded by Yalçin *et al*. (2015).

Metatech® WP that gave the lowest LT_{50} values is more virulent than the other evaluated EPFs. The time taken by EPFs to kill their host is in relation with their mode of action (Reda and Hatem, 2012; Mantzoukas and Eliopoulos, 2020). Spores of an EPF, when in contact with the host, go through a period of lethal infection that involves germination and growth before they cause death (Reda and hatem (2012). Klieber and Reineke (2016) reported significant reduction of feeding and damaging activity of the pest during this infection period.

4.5 Conclusion

The commercial formulations of entomopathogenic fungi (EPFs) used in this study: Metatech® WP [*Metarhizium anisopliae* (Metsch.) Sorok, Strain FCM Ar 23B3, 5 x 10^9 CFUs/g], Beauvitech® WP [*Beauveria bassiana* (Bals.) Vuill., Strain J25, 1 x 10^{10} CFUs/g], and Botanigard® ES [*Beauveria bassiana* (Bals.) Strain GHA, 2 x 10^{13} viable spores/1.14 L], at a dosage of 10^8 spores/mL, are pathogenic to *Tuta absoluta*. Metatech® WP exhibited the highest pathogenicity to *T. absoluta*, followed by Beauvitech® WP. The pathogenicity was judged by considering the mortality% and the time required to kill 50% of *T. absoluta* larvae (LT_{50}). Field evaluations should be carried out to verify laboratory efficacy of the EPFs under field conditions.

CHAPTER FIVE

BIOACTIVITY OF PLANT EXTRACTS AGAINST TOMATO LEAF MINER, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae)

Abstract

Tomato (*Solanum lycopersicum* L.) is economically and nutritionally important in Rwanda but its production is challenged by the tomato leaf miner (*Tuta absoluta* Meyrick). Synthetic insecticides which are the main method of its control have various drawbacks. Bioactivity of *Tephrosia vogelii*, *Tithonia diversifolia*, *Vernonia amygdalina,* and *Phytolacca dodecandra* aqueous extracts was evaluated against *T. absoluta* in laboratory. Leaflets with third instar larvae (3.85 - 5.65 mm) of *T. absoluta* in mines were collected from the established tomato field. Aqueous plant extracts were evaluated at a dose of 10% weight/volume. Sterile tap water and Azadirachtin 0.03% EC were used as negative and positive controls, respectively. Petri-dishes of 9 cm diameter (n=10) were used as bioassay arenas in a completely randomized design with four replications. Data on larval mortality were collected every 24 h for five days. Three bioassays were conducted on different dates. Results indicated that tested plant extracts exhibited a capacity to kill *T. absoluta* larvae in tomato leaf galleries with significant differences among them ($p < 0.0001$). The killing capacity increased with exposure time. At 24 h of exposure, *T. absoluta* larvae mortality was in a range of 35.0% - 37.5% for azadirachtin and 5.0% - 10.0% for *T. vogelii* while all other aqueous extracts had 0.0% mortality, except *V. amygdalina* which recorded 2.5% in bioassay one. In all bioassays, the lowest mortality recorded five days after treatment with *T. vogelii, T. diversifolia, V. amygdalina, P. dodecandra,* and azadirachtin was 32.2%, 2.8%, 2.5%, 20.5% and 97.5% while the highest mortality at this time was 35.1%, 10.6%, 13.3%, 24.9% and 100%, respectively. *Tephrosia vogelii and P. dodecandra,* which recorded higher efficacy as compared to the other local plants, should be advanced to field evaluation. The observed higher efficacy of azadirachtin to the Rwandan population of *T. absoluta* should also be confirmed under field conditions.

Keywords: Biopesticides, Botanicals, Insecticidal plants, *Phytolacca dodecandra*, *Solanum lycopersicum* L., *Tephrosia vogelii*, *Tithonia diversifolia*, *Vernonia amygdalina*

5.1 Introduction

Insect pests are one of the important causes of crop production losses all-over the world (Silva *et al*., 2011; Biondi *et al*., 2018). In particular, tomato leaf miner (*Tuta absoluta*), an invasive pest reported in Rwanda in 2015 (Uzayisenga *et al*., 2016), causes serious damage to tomato crop resulting in severe yield losses up to 100% (Desneux *et al*., 2010). The pest is now spread in all tomato production areas of Rwanda (Uzayisenga *et al*., 2016). Thus, it is vital to develop effective management strategies against this challenging pest.

The management of insect pests is crucial to ensure good crop productivity. Use of synthetic insecticides, the main method of insect control all-over the world (Senthil-Nathan, 2013), often results in pollution of ecosystems, apparition of resistant pest genotypes and new pests, and destruction of natural enemies among others (Macharia *et al*., 2009; Yalçin *et al*., 2015). Fortunately, various plants possess different chemicals recognised as secondary metabolites that have insecticidal properties and hence the potential of being used to manage various insect pests (Adeyemi, 2010; Shrivastava and Singh, 2014).

Research on botanical insecticides has been carried out for many years with the main goal to minimise the harmful effects of synthetic insecticides (Adeyemi, 2010). Azadirachtin is one of the widely known and successful examples of botanical insecticide discovery from plants (Mordue and Alasdair, 2000). It is effective against several pests and comparatively harmless to natural enemies than most of the commonly used synthetic insecticides (Gontijo *et al*., 2015). El-Ghany *et al*. (2016) obtained up to 92% *T. absoluta* larval mortality caused by azadirachtin. Tomé *et al*. (2013) also observed that azadirachtin is effective against *T. absoluta.* Although high efficacy was obtained with insecticides of plant origin like azadirachtin (Yalçin *et al*., 2015), they are relatively expensive. Therefore, evaluation and exploitation of extracts of locally available plants against *T. absoluta* are necessary because they are cheap, easy to prepare and contain multiple active components that impede the development of insect resistance (Braham *et al*., 2012).

Over two thousand plant species were reported to have insecticidal properties (Shivakumar *et al*., 2013) and studies have shown higher bioactivity of extracts from some plants such as *Acmella oleracea* (Asteraceae) *and Thymus vulgaris* (Lamiaceae) against *T. absoluta* larvae (Moreno *et al*., 2012; Nilahyane *et al*., 2012). Screening different plants to assess their potential against insect pests, including *T. absoluta*, would contribute to sustainable pest management while preserving the environment.

Locally available plant materials, such as *Tephrosia vogelii* (Leguminosae), *Tithonia diversifolia* (Asteraceae), *Vernonia amygdalina* (Asteraceae) and *Phytolacca dodecandra* (Phytolaccaceae), are known to exhibit the features required for an ideal botanical insecticide (Adeniyi *et al*., 2010; Olaitan *et al*., 2011; Mkenda *et al*., 2015; Raja *et al*., 2015) but their potential has not been evaluated against *T. absoluta*.

Crude extracts of the above-mentioned plants have shown the efficacy against various pests of different crops (Olaitan and Abiodun, 2011; Onunkun, 2012; Mkenda *et al*., 2015; Raja *et al*., 2015). Further exploration is needed to broaden their use in IPM of various crops in Rwanda. Furthermore, there is scarce information on their efficacy against *T. absoluta*. Finding the indigenous plant species with insecticidal properties along with a simple preparation technology would benefit more local farmers. The main aim of this study was to determine the bioactivity of four aqueous extracts from locally available plants (*T. vogelii*, *T. diversifolia*, *V. amygdalina,* and *P. dodecandra*) against *T. absoluta* in the framework of finding options for IPM of this pest in Rwanda.

5.2 Materials and methods

5.2.1 Collection of plant materials and preparation of extracts

Leaves of *T. diversifolia, T. vogelii, V. amygdalina,* and *P. dodecandra* (Plate 5. 1) were collected from various regions in Rwanda where they grow naturally.

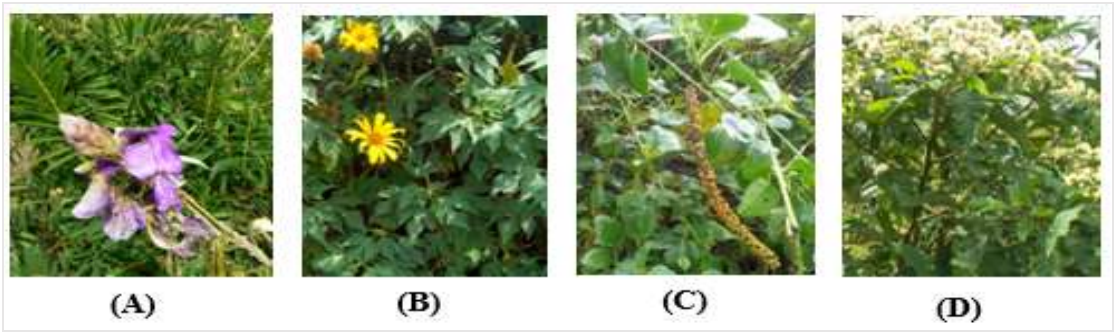

Plate 5.1 Plant species used for preparation of plant extracts: (A) *Tephrosia vogelii,* (B) *Tithonia diversifolia,* (C) *Phytolacca dodecandra*, (4) *Vernonia amygdalina*

The collected leaves were washed to remove sand, dust, and chemical contaminants; then dried under shade (to prevent denaturation of active chemicals) for two weeks and subsequently ground, using an electric grinder, into a fine powder which was packaged in biodegradable plastic bags. In a litre of boiled water, 100 g of powder for each plant species

were added separately. The powder was left in boiled water for 12 h and then filtered with a muslin cloth. The respective extracts were made to a volume of one litre using cold water to give 10% weight/volume (w/v). This solution was ready for use with no further dilution. Previous work had shown that 10% w/v of *T. vogelii* water extract was highly effective to certain insects (Adebayo *et al*., 2007).

5.2.2 Collection of *Tuta absoluta* larvae

Tomato leaflets containing *T. absoluta* larvae in galleries were collected from a tomato field, which was established in an area of the high occurrence of *T. absoluta* in Rwanda. This field was located in Rweru Sector of Bugesera District, Eastern Province, on latitude 02° 31′ 974″ S, longitude 030° 26′ 853″ E and at an altitude of 1342 m above sea level. Cloth bags were used to transport the collected leaflets from the field to the laboratory where they were used in bioassays for a maximum of a half-day (12 h) after their collection.

5.2.3 Laboratory bioassays

Three bioassays were carried out on different dates in the Biological Control Laboratory at Rwanda Agriculture and Animal Resources Development Board (Yan *et al.*, 2016). Each tomato leaflet, with one third instar larvae (3.85 - 5.65 mm) of *T. absoluta* in galleries, was dipped for three seconds (Cherif *et al*., 2018) in 10% w/v solution of respective plant extract and then positioned in a Petri-dish lined with three moistened filter paper discs. Each experimental unit was composed of ten Petri-dishes of 9 cm diameter, which received the same treatment. Apart from the four plant extracts, azadirachtin 0.03% EC (5 mL/L) and water were used as positive and negative controls, respectively. The Petri-dishes were sealed with parafilm and kept at a temperature of 25°C ± 2°C. Each bioassay was conducted in a completely randomised design with four replications.

5.2.4 Data collection and analysis

Mortality of *T. absoluta* larvae was recorded 24, 48, 72, 96 and 120 h after treatment application. Dead larvae outside the galleries were recognised by their inability to move back to the ventral position after being positioned on their dorsum. Larvae still inside the leaf galleries were recorded as dead when unable to respond to microscopic light or gentle touch by fine camel's hairbrush. The number of dead larvae per each treatment was brought to percentage mortality by considering the total number of larvae per treatment.

Mortality observed in negative control was used to correct mortality in plant extracts and positive control (azadirachtin) treatments using Schneider-Orelli's formula (Püntener, 1981). Collected data were checked for normality before analysis, using proc univariate procedures, and were found to be not normally distributed. Among different methods of data transformation tested, arcsine transformation was chosen and used before data analysis. Analysis of variance was carried out through the PROC GLM procedure. Means of the significantly different treatments ($p \leq 0.05$) were separated using Tukey's honestly significant difference (HSD) test. The level of significance was fixed at $\alpha = 0.05$. All these procedures were carried out using the Statistical Analysis System package (SAS) software version 9.2 (SAS Institute, 2010).

The statistical model fitted for this experiment was: $Y_{ij} = \mu + t_i + e_{ij}$
Where Y_{ij} = larva mortality, μ = overall mean, t_i = effect of i^{th} treatment of plant extract or control (i = 1, 2, 3, 4, 5) and e_{ij} = error term.

5.3 Results

The tested plant extracts (10% w/v) exhibited a capacity to kill *T. absoluta* larvae inside the leaf galleries. In all bioassays, statistical analysis revealed strong evidence ($p < 0.0001$) of significant difference in the bioactivity of evaluated plant extracts against *T. absoluta* larvae. The effect of the studied plant extracts on *T. absoluta* larvae increased progressively with the duration of exposure from 24 to 120 h after treatment application. Apart from *T. vogelii* that recorded the mortality range of 5.0% - 10.0% at 24 h after treatment application, all other aqueous extracts recorded 0.0% mortality at this time, except *V. amygdalina* which had 2.5% in bioassay one. At this time, mortality due to azadirachtin ranged between 35.0% and 37.5% in all bioassays. The lowest mortality recorded at 120 h of exposure to treatments in all bioassays was 32.2%, 2.8%. 2.5%, 20.5% and 94.5% while the highest mortality at this time was 35.1%, 10.6%, 13.3%, 24.9% and 100% for *T. vogelii, T. diversifolia, V. amygdalina, P. dodecandra,* and azadirachtin, respectively (Table 5.1).

Azadirachtin, which served as a positive control, resulted in higher larval mortality ($p < 0.0001$) as compared to all tested botanicals in the three bioassays from 24 to 120 h after treatment application. The efficacy of azadirachtin was followed by that of *T. vogelii*, which was not different from *P. dodecandra* during the period from 72 to 120 h following the application of treatments. *Tithonia diversifolia and V. amygdalina* were not significantly different in effect on *T. absoluta* larvae except during 120 h post-treatment in bioassay one.

Table 5.1 Mortality of *Tuta absoluta* larvae (mean ± SD) in tomato leaf galleries treated with aqueous plant extracts at 10% (w/v) and azadirachtin 0.03% EC (5 mL/L)

Treatments	*T. absoluta* larvae mortality (%)				
	24 h	48 h	72 h	96 h	120 h
Bioassay One					
T. vogelii	7.5±5.0 b*	15.0±5.8 b	18.3±4.9 b	28.5±5.7 b	35.1±8.1 b
T. diversifolia	0.0±0.0 c	0.0±0.0 c	2.5±5.0 b	2.8±5.6 c	2.8±5.6 d
V. amygdalina	2.5±5.0 bc	5.0±5.8 bc	5.3±6.1 b	5.6±6.4 c	11.8±0.8 c
P. dodecandra	0.0±0.0 c	10.0±0.0 c	10.6±0.6 b	11.5±0.7 bc	20.5±5.5 bc
Azadirachtin	35.0±5.8 a	77.5±12.6 a	81.4±19.1 a	94.5±6.4 a	100.0±0.0 a
p (α = 0.05)	p < 0.0001	p < 0.0001	p < 0.0001	p < 0.0001	p < 0.0001
Bioassay Two					
T. vogelii	5.0±5.8 b	18.1±5.5 b	25.6±5.2 b	26.4±6.3 b	32.2±7.4 b
T. diversifolia	0.0±0.0 b	2.5±5.0 c	5.0±5.8 c	5.0±5.8 c	10.6±8.2 c
V. amygdalina	0.0±0.0 b	2.5±5.0 c	7.5±5.0 bc	7.8±5.2 bc	13.3±4.4 bc
P. dodecandra	0.0±0.0 b	2.5±5.0 c	12.5±12.6 bc	21.1±1.3 b	21.7±9.1 bc
Azadirachtin	37.5±9.6.0 a	64.2±5.0 a	82.2±9.3 a	92.2±5.2 a	94.5±6.4 a
p (α = 0.05)	p < 0.0001	p < 0.0001	p < 0.0001	p < 0.0001	p < 0.0001
Bioassay Three					
T. vogelii	10.0±0.0 b	17.5±5.0 b	20.3±7.7 b	31.4±7.4 b	33.3±7.9 b
T. diversifolia	0.0±0.0 c	0.0±0.0 d	2.5±5.0 c	5.0±5.8 cd	5.3±6.1 c
V. amygdalina	0.0±0.0 c	0.0±0.0 d	2.5±5.0 c	2.5±5.0 d	2.5±5.0 c
P. dodecandra	0.0±0.0 c	7.5±5.0 c	18.1±5.5 b	18.3±4.9 bc	24.9±3.7 b
Azadirachtin	37.5±9.6 a	75.0±5.8 a	87.2±4.8 a	94.7±6.1 a	97.5±5.0 a
p (α = 0.05)	p < 0.0001	p < 0.0001	p < 0.0001	p < 0.0001	p < 0.0001

*Mean values followed by different letters in the same column are significantly different according to Tukey's test ($p \leq 0.05$). Each of the three bioassays represented a full experiment with three replications.

5.4 Discussion

The bioactivity of evaluated botanicals against larvae of *T. absoluta* is explained by the secondary metabolites produced by these plants, which have various modes of action (Gurjar

et al., 2012). *Tephrosia vogelii* contains rotenoid compounds, which are mitochondrial poisons that block the electron transport chain and prevent energy production. Rotenone is also known to be stomach and contact poison (Stoll, 2002). The bioactivity of *P. dodecandra* could be associated with the presence of phytochemicals such as saponins, alkaloids, sterols, triterpenoids, phenols, flavonoids, and glycosides (Qwarse *et al*., 2016). Insecticidal activity of *T. diversifolia* is due to some of the sesquiterpenes, diterpenes, monoterpenes, and alicyclic compounds in its leaves (Obafemi *et al*., 2006). Insecticidal properties of *V. amygdalina* are due to its content in alkaloids, flavonoids, saponins, tannins, phlobatannins, terpenoids and, cardiac glycosides (Adeniyi *et al*., 2010).

As compared to the findings of the present study, other authors reported higher efficacy of the evaluated botanicals against various insect pests. For instance, *T. vogelli* (10% w/v), was observed to be as effective as a chemical insecticide, Decis® EC25, in controlling *Maruca testularis*, *Ootheca mutabilis*, and *Zonocerus variegatus* on cowpea (Adebayo *et al*., 2007). Aqueous extracts of *T. vogelii* reduced significantly the pest population for *Maruca vitrata, Megalurothrips sjostedti*, and *Ripotortus dentipes* in cowpea field (Olaitan and Abiodun, 2011). Cold and hot water extracts of *P. dodecandra* at the rate of 10 g/100 mL caused 100% mortality of cabbage flea beetle *Phyllotreta cruciferae* in 24 h (Raja *et al*., 2015). Extracts of *P. dodecandra* were also effective against onion thrips under field conditions (Shiberu *et al*., 2012). Crude water extracts of *P. dodecandra* leaves resulted in mosquito (*Anophele gambiae*) egg mortality of more than 80% (Yugi and Kiplimo, 2017) with higher efficacy than neem and deltamethrin.

Furthermore, *T. diversifolia* extracts have been observed to be effective in field conditions against aphids and flower beetles of common bean (Mkenda *et al*., 2015). Aqueous leaf extract of *T. diverisifolia* caused 100% mortality of acrobat ant (*Crematogaster lineolata*), an insect pest of honeybees (Olufemi *et al*., 2015). The efficacy of 1.0% and 1.5% *T. diversifolia* hot water leaf extract was the same as a chemical insecticide, Permethrin, against oviposition of *Sitophilus zeamais* (Onekutu *et al*., 2015). *Vernonia amygdalina* was observed to be toxic to common bean aphids (Kawuki *et al*., 2005) and bean weevil, *Acanthoscelides obtectus* (Adeniyi *et al*., 2010). Water extracts of *V. amygdalina* leaves caused a reduction in the population of two flea beetles (*Podagrica uniforma* and *P. sjostedti*) at 55%, in okra (Onunkun, 2012).

Lower efficacy of plant extracts obtained in this study, as compared to the previous findings on other insects, could be due to the water extraction method used. This method was selected because the study was aiming to find indigenous plant species with insecticidal properties along with a simple preparation technology to benefit the local farmers. Furthermore, it is advised that initial screening of plants for possible bioactivity should begin by water (universal solvent) extracts; then extraction using different organic solvents can follow (Gurjar *et al*., 2012). Other methods of extractions would likely give better results. For example, Fan *et al*. (2011) demonstrated the relationship between the efficacy of plant extracts and the type of solvents used in extraction. The same authors reported that toxicity of *Piper nigrum* fruit extracts against second instar larvae of tobacco armyworm (*Spodoptera litura*) varied with the solvents used and decreased in the order of hexane (LD_{50}: 1.8 mg/g) > acetone (LD_{50}: 18.8 mg/g) > chloroform (LD_{50}: NA, the toxicity was very low). Similarly, Arora *et al*. (2017) obtained higher anti-feeding activity of *Paederia foetida* L. (Rubiaceae) against *Spodoptera litura* larvae with methanol extracts compared to water extracts.

Furthermore, Olufeni *et al*. (2015) reported that efficacy of N-hexane and methanol extracts from *T. diversifolia, Azadirachta indica, Ageratum conyzoides,* and *Carica papaya* against *Aethina tumida*, *Galleria mellonella*, and *Achroia grisella* was significantly higher than that of water extracts. Higher efficacy with organic solvents as compared to water could be due to their difference in polarity. Organic solvents such as N-hexane and methanol are less polar than water and this facilitates some organic compounds to be easily dissolved in them (Widyawati *et al*., 2014). Thus, further studies should be carried out using various solvents other than water to evaluate the potential of these indigenous plants against *T. absoluta*.

Lower efficacy obtained with evaluated aqueous extracts could also be attributed to the concentration used (10% w/v). According to Olaitan *et al*. (2011), levels of plant extract concentrations determine their efficacy against a given pest. In our research, the fixed concentration utilised was for screening purposes; higher concentration than 10% w/v would have resulted in higher mortality levels. Thus, further research should be continued with *T. vogelii* and *P. dodecandra*, which had higher efficacy than the other plants.

Higher efficacy recorded by azadirachtin in the present study corroborates the earlier findings by different researchers who obtained high mortality of *T. absoluta* larvae treated with this botanical insecticide. For instance, El-Ghany *et al*. (2016) obtained up to 92% *T. absoluta* larval mortality using azadirachtin. Similar results were obtained by Tomé *et al*. (2013).

Azadirachtin was proved to be effective against several other pests, such as *Brevicoryne brassicae*, *Sitophylus oryzae*, *Tribolium confusum,* and *Epilachna paenulata,* and comparatively harmless to natural enemies than most of the commonly used synthetic insecticides (Gontijo *et al*., 2015). The recorded azadirachtin efficacy may be attributed to its various modes of action such as enzyme inhibition, growth inhibition, feeding-deterrence and insecticidal activity among others (Senthil-Nathan, 2013). To the best of our knowledge, this is the first report of azadirachtin efficacy to the Rwandan population of *T. absoluta*; this efficacy should be confirmed under the field conditions of Rwanda.

5.5 Conclusion

The evaluated aqueous plant extracts displayed potential insecticidal properties and differed significantly against third instar larvae of *T. absoluta*. Further study should be carried out using the solvents other than water to evaluate the potential of these indigenous plants against *T. absoluta* in laboratory conditions. *Tephrosia vogelii* and *P. dodecandra*, which differed in efficacy from other studied aqueous plant extracts should be further evaluated under field conditions. Finally, the observed higher efficacy of azadirachtin to the Rwandan population of *T. absoluta* should be confirmed under the field conditions of Rwanda.

CHAPTER SIX

FIELD EFFICACY OF ENTOMOPATHOGENS AND PLANT EXTRACTS AGAINST *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae) INFESTING TOMATO IN RWANDA

Abstract

Following its outbreak in 2015, *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae) immediately became one of the major threats to the food chain in Rwanda. Sustainable management options are needed to address the situation. A field study was carried out to determine the efficacy of entomopathogens and plant extracts. Nine treatments were evaluated, including: entomopathogenic nematodes (EPNs) (*Steinernema* sp. RW14-M-C2a-3 and *Steinernema* sp. RW14-M-C2a-3), commercial formulations of entomopathogenic fungi (EPFs) [Metatech® WP: *Metarhizium anisopliae* (Metsch.) Sorok, Strain FCM Ar 23B3), Beauvitech® WP: *Beauveria bassiana* (Bals.) Vuill., Strain J25], plant extracts of *Tephrosia vogelii* and *Phytolacca dodecandra*, azadirachtin 0.03% EC, imidacloprid as positive control and water as negative control. Entomopathogens and azadirachtin significantly ($p < 0.05$) reduced leaf and leaflet damages as compared to the plant extracts and controls. However, leaf damage increased with time and reached the maximum level (100%) in 9 - 10 weeks after transplanting in all the treatments. The maximum leaflet damage obtained with entomopathogens and azadirachtin 10 weeks after transplanting varied between 59.7% and 74.7% while the positive control (imidacloprid) had 80.0% - 92.1%. The entomopathogens and azadirachtin, which exhibited higher field efficacy, should be included in integrated pest management of *T. absoluta* in Rwanda. Further studies are recommended to enhance the efficacy of the studied entomopathogens and to assess their efficacy in greenhouse conditions.

Keywords*: Beauveria bassiana, Metharizium anisopliae, Solanum lycopersicum* L. *Steinernema, Tephrosia vogelii,* Tomato leaf miner

6.1 Introduction

Control of pests is a pre-requisite for enhanced crop performance and subsequent production as pests can inflict severe damage resulting in total crop destruction (Desneux *et al*., 2010). Specifically, the tomato leaf miner, *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae) is a major challenge to tomato (*Solanum lycopersicum* L.) production in many parts of the world

(Biondi *et al*., 2018). Following its outbreaks in Rwanda in 2015, FAO (2015) declared this pest among the threats to the food chain in the country. *Tuta absoluta* larvae damage all parts of tomato plants, including stems, leaves, flowers, and fruits, resulting in interrupted crop growth and development (Biondi *et al*., 2018).

By feeding within the mesophyll, one larva can make many galleries, moving in and out the leaves (Gözel and Kasap, 2015). Studies reported a positive correlation between leaf and fruit infestations (Cocco *et al*., 2014). Up to 12 generations of this pest are possible under favourable conditions, which add to the invasive nature of this pest (Biondi *et al*., 2018). In the absence of proper management measures, yield losses inflicted by this pest can reach 100% of the total production (Desneux *et al*., 2010). Chemical control is the main option used by most African farmers to manage this pest. However, *T. absoluta* management remains a challenge mainly due to its mine-feeding habit, short development cycle, and acquisition of resistance to frequently used insecticides (Roditakis *et al*., 2015). This necessitates the search for sustainable alternatives.

Local isolates of EPNs (Yan *et al*., 2016) were demonstrated to be effective against white grubs in Rwanda (Kajuga *et al*., 2018), hence the need to broaden investigations of their efficacy against other economically important pests, including *T. absoluta*. On the other hand, the EPFs *Beauveria bassiana* (Bals.) Vuill. (Ascomycota: Hypocreales) and *Metarhizium anisopliae* (Metsch.) Sorok (Ascomycota: Hypocreales) have many advantages, including their efficiency in killing the host and the ability to attack all insect developmental stages (Schrank and Vainstein, 2010). In addition, since it was observed that insecticides of plant origin also can assist in *T. absoluta* management (Nilahyane *et al*., 2012), field efficacy of local insecticidal plants like *Tephrosia vogelii* and *Phytolacca dodecandra* against *T. absoluta* needs to be evaluated because they would be affordable to farmers.

In our previous studies, laboratory bioassays were carried out in Rwanda to evaluate the potential of using the three groups of biorational control agents: local EPN isolates, commercial formulations of EPFs and local plant extracts against *T. absoluta*. In each of the above groups, some agents demonstrated relatively higher efficacy. Since laboratory efficacy can only be partly transferred to field conditions (Lacey *et al*., 2015), field evaluation is mandatory for efficacy confirmation. The objective of the current study was to determine the field efficacy of the entomopathogens and plant extracts against *T. absoluta* infesting tomato in Rwanda.

6.2 Materials and methods

6.2.1 Study site and plant material

This study was conducted in Rweru Sector, Bugesera District, Eastern Province of Rwanda. GPS coordinates of the location are latitude 02º 32' 355" S, longitude 030º 26' 963" E and 1338 m of elevation above sea level. The average annual temperature and rainfall are 21.4ºC and 854 mm, respectively (Kabirigi *et al.*, 2017). The study was carried out on tomato (*Solanum lycopersicum* L.), which is the most preferred host of *T. absoluta*. The cultivar 'Roma' was selected because it is mostly cultivated by Rwandan farmers in open fields.

6.2.2 Treatments

Nine treatments (Table 6.1) were evaluated against *T. absoluta*: two local EPN isolates, two commercial formulations of EPFs, two local plant extracts (PEs), azadirachtin, imidacloprid, and water spray. The EPNs, EPFs, PEs, and azadirachtin were chosen because they performed well in previous laboratory bioassays and were recommended to be advanced at the field evaluation stage. Imidacloprid and water spray were added as positive and negative controls, respectively.

Table 6.1 Treatments used in the field experiment to control *Tuta absoluta*

Designation	Treatment description	Type of treatment
T1	*Steinernema* sp. RW14-M-C2a-3	Entomopathogenic nematode
T2	*Steinernema* sp. RW14-M-C2b-1	Entomopathogenic nematode
T3	Metatech® WP (*M. anisopliae*, Strain FCM Ar 23B3, 5×10^9 CFU/g)	Entomopathogenic fungi
T4	Beauvitech® WP (*B. bassiana*, Strain J25, 1×10^{10} CFU/g)	Entomopathogenic fungi
T5	*Tephrosia vogelii*	Local plant extracts
T6	*Phytolacca dodecandra*	Local plant extracts
T7	Azadirachtin 0.03% EC (Nimbecidine)	Botanical insecticide
T8	Imidacloprid (Confidor SL 200)	Neonicotinoid insecticide
T9	Water (negative control)	-

Entomopathogenic nematodes

Two EPNs used in this study (Table 6.1) were obtained from the Biological Control Laboratory – EPN Production Facility of Rwanda Agriculture and Animal Resources Development Board. These EPNs were isolated in the year 2014 from Musanze District, Northern Province, Rwanda, in a field of banana intercropped with sorghum and pumpkin (Yan *et al*., 2016). To obtain the required number of EPNs to be used on experimental plots, they were mass-produced following the *in-vivo* method using *Galleria mellonella* larvae (Kaya and Stock, 1997). Upon harvesting, the infective juveniles (IJs) were rinsed in distilled water and stored at 7^{o}C for not more than 7 days before their use (Mahmoud, 2016).

On the day of application in the field, the EPNs were checked for viability using a stereomicroscope (60× magnification) after acclimatization for one hour at room temperature (± 19^{o}C). The EPNs were used when more than 90% of IJs were moving actively (Kajuga *et al*., 2018). After checking their viability, the EPNs were counted and adjusted to the required concentration of 5 x 10^{9} IJs/ha **(**Gözel and Kasap, 2015; Kamali *et al*., 2018). The aqueous suspension of IJs for each EPN was then transferred into sponges packed in plastic bags, transported in a cool box to the field and used the same day at dusk (Yan *et al*., 2016). At the time of application, the sponges containing EPNs were diluted in water for the EPNs to get out and then the required volume was made up by adding water.

Entomopathogenic fungi

Two commercial formulations of EPFs used in this study (Table 6.1) were manufactured by Dudutech Division, Flamingo Horticulture (K) Ltd, Naivasha, Kenya. Before their application in field, their viability was checked by culturing them on Potato Dextrose Agar media and incubating at 25^{o}C ± 1^{o}C for 7 days (Youssef, 2015). The EPFs were observed under light microscopy (×40 magnification), to ensure that more than 95% of spores had germinated to proceed with them to the field for application.

Plant extracts

Two local plant extracts used in this study were obtained from the leaves of *T. vogelii* and *P. dodecandra* collected from Huye District, Southern Province of Rwanda. Upon collection, the leaves of each species were washed, dried under shade and ground into a fine powder using an electric grinder. The obtained powder was packed in biodegradable plastic bags.

Before field application, extraction for each plant species was carried out by adding 150 g of powder in one litre of boiled water, immediately after its removal from heat, and keeping it for 12 h. Thereafter, filtration was carried out using a muslin cloth and the extracts were made to a volume of one litre each using cold water to give the concentration of 15% w/v.

6.2.3 Trials establishment and maintenance

Two field trials, from here onward referred to as "Trial one and Trial two" were established on 3rd April 2019 and 28th June 2019, respectively. Before planting, the field was ploughed twice at 15 days' interval and incorporated with cow manure, 20 t/ha. The experiment was laid out in a randomized complete block design with three replications. Each experimental unit was 3 m long and 2 m wide to accommodate 24 plants spaced at 0.5 m × 0.5 m. The plots and blocks were separated by a 1.5 m wide path to avoid the drifting effect of the treatments. Thirty days old seedlings were transplanted into the plots and mulched with dry grass. Apart from insecticide application, other practices like watering, weeding, pruning to four branches per plant, fertilizer, and fungicide application were carried out uniformly in all plots. Due to severe infestation by *T. absoluta* in the area of study, the trials relied on natural infestation (Sohrabi *et al*., 2017) and were established next to old tomato fields infested with *T. absoluta.*

6.2.4 Application of treatments

The application of treatments started one week after transplanting and was done during evening hours, slightly before sunset (around 4:30 pm), to avoid the harmful effect of sunlight on the treatments (Gözel and Kasap, 2015). For each treatment, the spray volume was 1000 L/ha (Brusselman *et al*., 2012) using a knapsack sprayer and the application was done at weekly intervals. The dosages used were 5 x 10^9 IJs/ha for EPNs (Gözel and Kasap, 2015; Kamali *et al*., 2018), 250 g/ha for EPFs (from product labels), 15% w/v for local plant extracts, 5 mL/L for azadirachtin 0.03% EC, and 1mL/L of water for imidacloprid. Continuous agitation was done during treatment application to prevent precipitations.

6.2.5 Data collection and analysis

Data were collected on five plants in the middle of each plot and the averages per plant were computed. Observations started two weeks after transplanting and were done every week. Leaf damage was assessed as the percentage of leaves affected (mined) by *T. absoluta*; while leaflet damage was evaluated as the percentage of leaflets affected by *T. absoluta* on three leaves from the middle third leaf of each of the five selected plants (Cocco *et al*., 2014).

The collected data were entered in SAS software version 9.2 (SAS Institute, 2010) where the analysis was performed. Normality checking was carried out and the appropriate transformation was done to fulfil the assumptions of analysis of variance. Data on leaf damage were log-transformed before analysis, except in trial one where the data recorded on week two were square-root transformed, the ones recorded on weeks eight, nine and ten were not transformed; while in trial two the data of weeks seven, eight, nine and ten were not transformed. Data on leaflet damage were log-transformed, except the data of two weeks after transplanting, which were square root-transformed in trial one. Data were subjected to analysis of variance to determine the effect of treatments on studied parameters. The means for statistically different treatments were separated using Tukey's honestly significant difference (HSD) test at $p \leq 0.05$.

The statistical model fitted for this experiment was: $Y_{ij} = \mu + \beta i + t_j + e_{ij}$
Where Yij = observation on tomato, μ = overall mean, βi = effect i^{th} block (i = 1, 2, 3), tj = effect j^{th} treatment (j = 1, 2, 3, 4, 5, 6, 7, 8, 9) and e_{ij}= error term

6.3 Results

6.3.1 Leaf damage

Leaf damage (%) increased with time and reached the maximum level (100%) in nine to ten weeks after transplanting for all the treatments in trials one and two There was no significant difference in leaf damage among the treatments on weeks two, nine, and ten after transplanting in both trials, but also on week eight in trial two. During the other times of observation, the general trend was that the EPNs (*Steinernema* sp. RW14-M-C2a-3 and *Steinernema* sp. RW14-M-C2b-1), EPFs (Metatech® WP: *M. anisopliae*, Strain FCM Ar 23B3, and Beauvitech® WP: *B. bassiana*, Strain J25) and azadirachtin recorded lower leaf damage and were not significantly different from each other in trials one and two. They all significantly ($p < 0.05$) reduced leaf damage as compared to the controls (imidacloprid and water). Their efficacy was similar to *T. vogelii*, but the later produced significantly higher leaf damage during weeks six and seven in trial one, and week four in trial two. *Phytolacca dodecandra* was not significantly different from the controls (Table 6.2).

6.3.2 Leaflet damage

There was a significant difference in leaflet damage (%) among treatments ($p < 0.05$), except on week two for both trials (Table 6.3).

Table 6.2 Leaf damage (%) (mean ± SD) by *Tuta absoluta* on tomato cv. Roma crop treated with entomopathogens and plant extracts

	2 WAT	3 WAT	4 WAT	5 WAT	6 WAT	7 WAT	8 WAT	9 WAT	10 WAT
Trial One									
T1	3.5 ± 3.1 a	7.5 ± 4.0 b	16.2 ± 1.7 d	36.2 ± 3.7 c	51.9 ± 2.3 c	71.0 ± 0.8 d	83.3 ± 2.2 d	97.9 ± 2.2 a	100.0 ± 0.0 a
T2	3.6 ±3.1 a	7.9 ± 0.3 ab	17.7 ± 0.5 cd	38.5 ± 2.0 cb	57.2 ± 3.4 c	70.9 ± 1.2 d	83.9 ± 1.5 d	97.9 ± 2.1 a	99.3 ± 1.2 a
T3	3.5 ±3.1 a	7.3 ± 0.2 ab	16.1 ± 2.2 d	39.0 ± 1.5 cb	52.8 ± 3.1 c	73.4 ± 2.3 d	86.2 ± 1.4 cd	97.9 ± 0.1 a	100.0 ± 0.0 a
T4	3.7 ±3.2 a	10.0 ± 1.5 ab	19.5 ±1.1bcd	37.2 ± 2.8 cb	59.5 ± 4.6 bc	75.1 ± 3.1 cd	86.9 ± 3.8 cd	98.6 ± 1.2 a	100.0 ± 0.0 a
T5	5.4 ±0.6 a	12.3 ± 5.5 ab	22.2 ± 1.9 bc	44.2 ± 2.3 b	68.9 ± 0.9 ab	80.8 ± 1.8 bc	89.0±2.9 bcd	98.6 ± 1.3 a	100.0 ± 0.0 a
T6	5.3 ±5.3 a	13.2 ± 5.1 ab	24.9 ± 2.9 ab	63.0 ± 2.7 a	74.8 ± 1.7 a	85.4 ± 1.6 ab	92.7±1.7 abc	99.3 ± 1.2 a	100.0 ± 0.0 a
T7	3.4 ±2.9 a	8.4 ± 1.6 ab	17.5 ± 2.7 cd	39.0 ± 1.0 cb	55.1 ± 3.3 c	71.5 ± 1.7 d	83.0 ± 1.9 d	97.4 ± 1.0 a	99.4 ± 1.1 a
T8	3.2 ±2.8 a	14.0 ± 3.1 ab	24.3 ± 1.9 ab	61.4 ± 3.5 a	75.3 ± 5.6 a	87.6 ± 2.1 a	94.4 ± 2.5 ab	99.3 ± 1.2 a	100.0 ± 0.0 a
T9	3.5 ±3.1 a	18.2 ± 3.5 a	30.2 ± 1.2 a	71.9 ± 2.2 a	81.1 ±2.9 a	91.9 ± 2.0 a	97.1 ± 2.5 a	99.3 ± 1.2 a	100.0 ± 0.0 a
CV	51.63	14.49	3.49	1.56	1.35	0.60	2.84	1.49	0.56
p	0.9963	0.0355	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.6473	0.5934
Trial Two									
T1	6.9 ±3.1 a	11.4 ± 4.1 b	18.9 ± 6.7 c	45.2 ± 6.5 b	63.9 ± 3.0 d	81.9 ± 0.3 c	98.3 ± 2.6 a	100 ± 0.0 a	100 ± 0.0 a
T2	7.0 ±2.6 a	14.7 ± 3.9 ab	21.8 ± 3.4 c	42.0 ± 6.8 b	66.2 ± 2.3 d	83.2 ± 1.3 c	98.4 ±1.6 a	100 ± 0.0 a	100 ± 0.0 a
T3	7.0 ±2.6 a	18.0 ± 3.5 ab	20.3 ± 4.0 c	47.9 ± 2.2 b	70.5 ± 1.6 cd	83.3 ± 1.2 c	100.0 ± 0.0 a	100 ± 0.0 a	100 ± 0.0 a
T4	5.5 ±0.4 a	15.1 ± 4.3 ab	23.9 ± 5.6 bc	47.3 ± 4.1 b	72.9±5.3 bcd	83.9 ± 0.2 c	99.5 ± 0.9 a	100 ± 0.0 a	100 ± 0.0 a
T5	6.9 ±3.2 a	21.0 ± 2.2 ab	44.6 ± 6.5 ab	56.0 ± 7.5 b	71.5 ± 4.4 cd	88.3 ± 3.8 bc	100.0 ± 0.0 a	100 ± 0.0 a	100 ± 0.0 a
T6	7.0 ±3.0 a	27.1 ± 3.1 a	53.9 ± 1.7 a	80.1 ± 2.1 a	85.2 ± 4.1 ab	95.2 ± 6.3 ab	100.0 ± 0.0 a	100 ± 0.0 a	100 ± 0.0 a
T7	6.9 ±2.7 a	17.0 ± 7.0 ab	23.4 ± 8.5 c	46.3 ± 7.4 b	67.5 ± 2.3 d	83.2 ± 2.5 c	96.8 ± 2.9 a	100 ± 0.0 a	100 ± 0.0 a
T8	7.2 ±2.8 a	28.2 ± 8.2 a	53.2 ± 3.2 a	78.5 ± 3.7 a	83.3±8.6 abc	91.8 ± 2.7 ab	100.0 ± 0.0 a	100 ± 0.0 a	100 ± 0.0 a
T9	6.9 ±3.1 a	29.3 ± 7.5 a	60.9 ± 5.9 a	84.9 ± 2.6 a	83.8 ± 3.7 a	96.6 ± 3.2 a	100.0 ± 0.0 a	100 ± 0.0 a	100 ± 0.0 a
CV	20.62	9.73	6.66	2.85	1.39	3.05	1.38	0	0
p	0.9992	0.0070	<.0001	<.0001	<.0001	<.0001	0.1050	-	-

T1: *Steinernema* sp. RW14-M-C2a-3, T2: *Steinernema* sp. RW14-M-C2b-1, T3: Metatech®WP (*Metarhizium anisopliae*, Strain FCM Ar 23B3), T4: Beauvitech® WP (*Beauveria bassiana*, Strain J25), T5: *Tephrosia vogelii*, *T6: Phytolacca dodecandra*, T7: azadirachtin 0.03% EC, T8: imidacloprid, T9: water; Means followed by the same letter (s) are not significantly different according to Tukey's test ($p \leq 0.05$).

Table 6.3 Leaflet damage (%) (mean ± SD) by *Tuta absoluta* on tomato cv. Roma crop treated with entomopathogens and plant extracts

	2 WAT	3 WAT	4 WAT	5 WAT	6 WAT	7 WAT	8 WAT	9 WAT	10 WAT
Trial One									
T1	2.9 ± 2.6 a	4.2 ± 0.4 b	8.5 ± 0.6 d	13.4 ± 0.2 c	18.4 ± 1.4 c	26.1 ± 2.5 d	37.6 ± 2.8 d	48.8 ± 2.2 d	59.7 ± 4.8 c
T2	3.0 ± 2.6 a	4.7 ± 0.5 ab	8.9 ± 0.6 d	14.3 ± 1.0 c	20.1 ± 2.2 c	27.9 ± 3.8 cd	38.7 ± 1.4 d	51.5 ± 1.4 bcd	64.1 ± 2.6 c
T3	2.9 ± 2.6 a	5.5 ± 1.2 ab	9.4 ± 0.7 d	13.7 ± 0.5 c	19.3 ± 1.2 c	26.9 ± 3.0 d	38.5 ± 2.2 d	54.9 ± 0.8 bc	63.5 ± 0.7 c
T4	2.2 ± 3.8 a	5.8 ± 0.8 ab	9.3 ± 0.6 d	15.0 ± 1.1 c	21.1 ± 0.8 c	26.4 ± 0.3 d	39.5 ± 1.7 d	54.5± 3.6 bcd	66.0 ± 4.4 c
T5	3.0 ± 1.5 a	5.8 ± 0.8 ab	11.2 ± 0.8 c	18.0 ± 0.8 b	27.0 ± 1.1 b	35.7 ± 2.4 bc	49.3 ± 3.1 c	56.9 ± 2.8 b	71.0 ± 3.2 bc
T6	2.8 ± 2.6 a	7.8 ± 0.9 a	14.4 ± 0.5 b	20.2 ± 0.7 b	26.7 ± 1.6 b	39.6 ± 1.9 ab	60.2 ± 2.7 ab	71.6 ± 1.0 a	81.1 ± 1.5 ab
T7	2.4 ± 2.3 a	4.5 ± 1.3 b	8.2 ± 0.8 d	13.4 ± 0.3 c	21.0 ± 2.2 c	27.7 ± 4.0 cd	38.5 ± 2.0 d	49.8 ± 2.8 cd	63.7 ± 5.7 c
T8	2.7 ± 2.3 a	7.1 ± 1.4 ab	12.7 ± 0.5 cb	20.5 ± 1.3 b	28.6 ± 2.6 ab	37.9 ± 2.8 ab	58.5 ± 3.7 bc	70.6 ± 0.1 a	80.0 ± 4.9 ab
T9	2.6 ± 2.5 a	8.2 ± 2.1 a	17.5 ± 0.7 a	26.9 ± 1.0 a	35.0 ± 1.3 a	47.5 ± 4.6 a	70.0 ± 5.7 a	76.4 ± 2.6 a	90.4 ± 7.2 a
CV	54.4	11.05	2.39	1.75	2.25	2.77	1.60	1.00	1.51
P	0.9997	0.0042	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
Trial Two									
T1	2.2 ± 0.2 a	3.9 ± 0.9 bc	6.4 ± 1.4 b	17.7 ± 2.3 c	21.1 ± 2.8 c	28.8 ± 1.6 c	41.8 ± 3.3 c	55.7 ± 2.6 c	68.0 ± 1.5 b
T2	2.1 ± 0.2 a	4.0 ± 0.3 bc	7.0 ± 0.9 b	17.0 ± 1.3 c	21.2 ± 1.1 c	30.1 ± 3.5 c	44.5 ± 1.5 bc	57.4 ± 3.0 c	70.0 ± 2.1 b
T3	1.9 ± 0.3 a	4.7±0.4 abc	6.8 ± 0.2 b	16.7 ± 0.9 c	22.7 ± 3.2 c	32.5 ± 0.7 bc	43.1 ± 0.5 bc	57.0 ± 1.7 c	70.8 ± 1.7 b
T4	2.0 ± 0.2 a	5.0±0.9 abc	6.7 ± 1.2 b	18.8 ±2.7 bc	23.0 ± 2.7 c	32.4 ± 1.8 bc	44.8 ± 3.9 bc	59.0 ± 2.4 bc	71.3 ± 3.6 b
T5	2.1 ± 0.3 a	3.7 ± 1.0 c	6.9 ± 0.4 b	24.8 ±1.1 ab	28.4 ± 1.4 cb	37.8 ± 2.6 b	50.1 ± 3.4 b	64.0 ± 1.3 b	74.7 ± 2.6 b
T6	2.0 ± 0.2 a	5.6 ± 0.8 ab	14.5 ± 1.5 a	28.6 ± 2.5 a	35.0 ± 3.3 ab	54.2 ± 1.8 a	71.4 ± 4.1 a	82.4 ± 1.6 a	92.4 ± 2.4 a
T7	2.1 ± 0.2 a	3.6 ± 0.2 c	6.7 ± 0.2 b	17.3 ± 1.9 c	21.9 ± 3.1 c	30.3 ± 1.8 c	43.4 ± 3.4 bc	56.0 ± 1.5 c	70.1 ± 0.6 b
T8	2.3 ± 0.7 a	5.3±0.6 abc	15.2 ± 0.2 a	27.1 ± 2.1 a	36.0 ± 3.0 ab	53.1 ± 1.8 a	70.8 ± 1.1 a	81.7 ± 2.0 a	92.1 ± 3.0 a
T9	2.1 ± 0.7 a	6.5 ± 0.6 a	19.1 ± 0.8 a	31.2 ± 2.7 a	42.6 ± 0.8 a	56.4 ± 2.2 a	73.8 ± 1.3 a	84.8 ± 3.8 a	95.6 ± 3.8 a
CV	9.67	9.97	5.11	3.30	3.23	1.76	1.40	0.89	0.75
P	0.9708	0.0013	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001

T1: *Steinernema* sp. RW14-M-C2a-3, T2: *Steinernema* sp. RW14-M-C2b-1, T3: Metatech®WP (*Metarhizium anisopliae*, Strain FCM Ar 23B3), T4: Beauvitech® WP (*Beauveria bassiana*, Strain J25), T5: *Tephrosia vogelii, T6: Phytolacca dodecandra*, T7: Azadirachtin 0.03% EC, T8: Imidacloprid, T9: Water; Means followed by the same letter (s) are not significantly different according to Tukey's test ($p \leq 0.05$)

All entomopathogens (EPNs and EPFs) and azadirachtin recorded lower leaflet damages, which were not significantly different from one another. *Tephrosia vogelii* recorded slightly higher leaflet damage as compared to the entomopathogens and azadirachtin, but it was not significantly different from them or one of them during weeks seven, nine, and ten in trial one and during all weeks in trial two. Higher leaflet damage was observed in plots treated with *P. dodecandra* and controls, which were not significantly different from one another for most of the weeks after transplanting (Table 6.3).

6.4 Discussion

The potential of using the studied entomopathogens and plant extracts against *T. absoluta* had been previously obtained in laboratory conditions (Chapters three, four and five); field efficacy confirmation was, therefore, the subsequent step because laboratory efficacy does not always ascertain field efficacy (Lacey *et al.*, 2015). The evaluated EPNs, EPFs, and azadirachtin exhibited higher efficacy against *T. absoluta* under the field conditions of Rwanda as they significantly reduced leaf and leaflet damages as compared to controls (imidacloprid and water spray). Thus, in addition to environmental protection, these biocontrol agents can contribute to reducing the population of *T. absoluta*.

Higher field efficacy of EPNs against *T. absoluta* was also obtained by Shams-El-Din *et al*. (2014) and Gözel and Kasap (2015) under field conditions, and by Battalla-Carrella *et al.* (2010) in pot experiments. Moreover, EPNs have already been used on other crops as foliar applications under field conditions against various other insect pests (Mahmoud, 2016). The observed EPNs' efficacy could be linked to their ability to penetrate leaf galleries, formed by *T. absoluta* larvae, where they get protection against harsh environmental conditions (Battalla-Carrella *et al.*, 2010; Kamali *et al.*, 2018). It also seems that *T. absoluta* larvae might have served as ideal hosts, upon which IJs of the EPNs could multiply while preparing to attack other larvae. The ability of IJs of EPNs to survive and multiply in different hosts was reported by Belien (2018). Furthermore, higher efficacy of EPNs belonging to the *Steinernema* genus could be due to bacteria associated with their genus (*Xenorhabdus*) and host scavenging behaviour, ambusher strategy, by which they diligently wait for their host (Mahmoud, 2016).

Mahmoud (2016) and Belien (2018) reviewed different formulations that can be used to boost the efficacy of EPNs under field conditions. These include: vermiculite, clay, polyacrylamide gels, water-dispersible granules, peat, surfactants, polymers, and capsules among others.

This means that the EPNs' efficacy obtained in this study can be improved further by adopting a specific formulation, as also evidenced by other researchers (Jaffuel *et al*., 2020; Makirita *et al*., 2020; Saleh *et al*., 2020). For instance, Lacey *et al*. (2010) obtained enhanced efficacy of *S. feltiae* against the larvae of codling moth (*Cydia pomonella* L.) by applying wood flour foam as an anti-desiccant agent. Enhanced efficacy of *S. carpocapsae* was also obtained against the lesser peach tree borer (*Snanthedon pictipes*) when a sprayable gel, Barricade®, was sprayed after its application (Shapiro-Ilan *et al*., 2010).

Similary, Van Damme *et al*. (2016) obtained higher leaf deposition by IJs of *S. feliae* and observed non-significantly different mortality rates of *T. absoluta* larvae with full, half and quarter dosage of the EPN combined with surfactants, Addit or Silwet. However, Beck *et al*. (2013) and Makirita *et al*. (2020) showed that some adjuvants and formulations can have a negative effect on the viability of EPNs. Further studies are therefore needed to determine the formulation (s) that can boost the efficacy of the studied EPN isolates. Other factors that could affect the efficacy of EPNs include application equipment, relative humidity or high moisture levels on plants (Beck *et al*., 2013; Mahmoud, 2016; Saleh *et al*., 2020).

In accordance with the results obtained on the efficacy of the studied EPFs against *T. absoluta*, Tadele and Emana (2017) also reported high mortality of *T. absoluta* larvae under laboratory and glasshouse conditions in Ethiopia using *B. bassiana* and *M. anisopliae.* The higher efficacy of *B. bassiana* was also observed against *Stomphastis thraustica,* the leaf miner of jatropha plant (Moawad *et al*., 2017). The higher efficacy of *M. anisopliae* as compared to chemicals was also reported by Ansari *et al*. (2007) against pupae of the Western flower thrips (*Frankilinea occidentalis*). According to Klieber and Reineke (2016), *T. absoluta* larvae mortality inflicted by EPFs takes place at the late developmental stage (Mantzoukas and Eliopoulos, 2020), but during the infection period, the feeding activity of larvae is reduced progressively until death. This is the reason why with the use of EPFs, crop damages can still occur but the long-term effect is expected through reduction of population density (Klieber and Reineke, 2016). A similar situation was observed in this study where leaf and leaflet damages could be recorded but were significantly lower than the controls, which suggests that the efficacy obtained with the studied EPFs should not be underrated.

The observed efficacy of *B. bassiana* can be explained by its ability to exhibit epiphytic and endophytic activity against various insect pests, including *T. absoluta* (Klieber and Reineke, 2016; Nishi *et al*., 2020). Through the endophytic activity, spores also enter in plant tissues

and can persist for many months so that the control of pest progenies is guaranteed through the ingestion of spore-colonised tissues by a pest (Allegrucci *et al*., 2017). The endophytic behaviour of *B. bassiana* is of great importance because it allows for the persistence of fungi propagules which, could otherwise be killed by unfavourable environmental factors (Klieber and Reineke, 2016). Virulence of *M. anisopliae* could be explained by the presence of numerous proteases (more than 14) used to penetrate the host cuticle (Schrank and Vainstein, 2010). In addition, the strains of *M. anisopliae* produce a higher quantity of dextruxins, toxins known for the most virulence factors (Schrank and Vainstein, 2010).

Possibilities also exist to enhance further the efficacy of EPFs by manipulating the formulations. For instance, spores of EPFs do not dissolve in water (hydrophobic) and thus oil-based formulations have been reported to improve their dispersion, protect spores from UV radiation and desiccation, and finally enhance their persistence and efficacy (Murugasridevi *et al*., 2017). Therefore, more studies are needed with the commercial formulations tested in this study in order to enhance their efficacy. Since the EPFs used are the commercial formulations (Metatech® WP and Beauvitech® WP) currently recommended against other pests, they can be easily registered and used in IPM of *T. absoluta* in Rwanda.

Azadirachtin's field efficacy against the Rwandan population of *T. absoluta* under field conditions is now reported for the first time. These findings agree with other researchers (Tomé *et al*., 2013) who also obtained higher efficacy of azadirachtin against *T. absoluta*. Similarly, Nadeem *et al*. (2015) reported higher field efficacy of azadirachtin against jassid (*Amrasca devastans*) and whitefly (*Bemisia tabaci*) which was as good as lambda-cyhalothrin 2.5 EC in Okra. Debashri and Tamal (2012) reported azadirachtin to be also effective against several economically important pests, such as pod borer (*Helicoverpa armigera)*, cabbage aphid (*Brevicornye brassicae*), and potato tuber moth (*Phthorimaea operculella*). Azadirachtin was reported to act as oviposition-deterrent, repellent, anti-feeding, growth, and development inhibitor (Senthil-Nathan, 2013). Tomé *et al*. (2013) reported the ability of azadirachtin to harm the development of larvae and compromise their survival for *T. absoluta* and also several other leaf miners such as coffee leafminer (*Leucoptera coffeella*) and Diptera leafminers (*Liriomyza* spp.). The field efficacy observed in this study could be due to the combination of these modes of action which compromise the overall pest activity of the pest.

Tephrosia vogelii recorded relatively medium to lower field efficacy as compared to the entomopathogens and azadirachtin. This efficacy can be attributed to the presence of

rotenoids (Stevenson *et al*., 2012). Previous studies also reported higher efficacy of some other plant extracts against *T. absoluta*. For instance, leaf extracts of *Thymus vulgaris* L. and seed extracts of *Ricinus communis* L. caused up to 95% and 58% of *T. absoluta* larval mortality, respectively (Nilahyane *et al*., 2012). The bioactivity of plant extracts against various pests is due to secondary metabolites (Stevenson *et al*., 2012). The reduced field efficacy of plant extracts used in this study as compared to the entomopathogens and azadirachtin may be partly due to the quick degradation of active components when exposed to sunlight (James *et al*., 2019; Isman, 2020). Other factors affecting the efficacy of plant extracts, namely extraction method and concentration (w/v), were discussed in Chapter five. Lower efficacy of imidacloprid could be due to the ability of *T. absoluta* to develop resistant strains to frequently used pesticides (Roditakis *et al*., 2015). The resistance of *T. absoluta* was reported against the pesticides belonging to different chemical classes, including organophosphates, pyrethroids, spinosyns, avermectins, cartap, benzoylureas, indoxacarb, and diamides, among others (Guedes *et al*., 2019). This indicates that relying on synthetic pesticides is not a sustainable solution for the management of *T. absoluta*.

6.5 Conclusion

The studied entomopathogens and plant extracts exhibited significant field efficacy against *T. absoluta*. Higher efficacy was obtained with *Steinernema* sp. RW14-M-C2a-3, *Steinernema* sp. RW14-M-C2b-1, Metatech®WP (*Metarhizium anisopliae*, Strain FCM Ar 23B3), Beauvitech® WP **(***Beauveria bassiana*, Strain J25), and azadirachtin 0.03% EC as compared to plant extracts (*T. vogelii* and *P. dodecandra*). The entomopathogens and azadirachtin that exhibited relatively higher field efficacy should be included in integrated pest management of *T. absoluta* in Rwanda. Further studies on formulations for the entomopathogens are recommended to enhance their efficacy. The efficacy of the studied treatments should also be evaluated in greenhouse conditions.

CHAPTER SEVEN

GROWTH, YIELD AND FRUIT QUALITY OF TOMATO AS AFFECTED BY MANAGEMENT OPTIONS FOR TOMATO LEAF MINER, *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae)

Abstract

The current study evaluated the effect of entomopathogens and plant extracts, used against *T. absoluta*, on growth, yield, and quality of tomato. Two field trials were carried out in a randomised complete block design replicated thrice. The treatments were *Steinernema* sp. RW14-M-C2a-3, *Steinernema* sp. RW14-M-C2a-3, Metatech® WP (*Metarhizium anisopliae*, Strain FCM Ar 23B3), and Beauvitech®WP (*Beauveria bassiana*, Strain J25) as entomopathogens, *Tephrosia vogelii* and *Phytolacca dodecandra* as plant extracts, and azadirachtin 0.03% EC (Nimbecidine). In addition, imidacloprid (Confidor SL 200) and water were included as positive and negative controls, respectively. These treatments significantly ($p < 0.05$) influenced tomato growth and yield parameters in both trials one and two. Plant height and stem diameter were significantly higher in plots treated with entomopathogens and azadirachtin. The highest yield parameters were recorded with the entomopathogens and azadirachtin, which were not significantly different in most cases. The increase in weight of healthy fruits per plant (average of two trials) as compared to the negative control (water spray) was 11.4, 10.8, 10.1, 9.6, 3.96, 2.2, 11.7 and 2.4 folds for *Steinernema* sp. RW14-M-C2a-3, *Steinernema* sp. RW14-M-C2a-3, Metatech® WP, Beauvitech WP, *T. vogelii*, *P. dodecandra*, azadirachtin, and imidacloprid, respectively. However, there was no significant difference in the number of leaves per plant and fruit quality parameters. The values obtained (average of two trials) for fruit quality parameters are 3.3 kgF/cm^2 for fruit firmness, 4.3 ^{o}Brix for total soluble solids, 8.2 mg/100 g of fruit for beta-carotene, 5.5 mg/100 g of fruit for lycopene and 14.5 mg/100 g of fruit for ascorbic acid. The entomopathogens and azadirachtin, which exhibited a capacity to enhance tomato growth and reduced yield losses due to *T. absoluta*, are recommended to be included in integrated pest management of this pest in Rwanda.

Key words: Azadirachtin, *Beauveria bassiana,* Integrated pest management, *Metarhizium anisopliae, Phytolacca dodecandra, Solanum lycopersicum* L., *Steinernema, Tephrosia vogelii.*

7.1 Introduction

The increasing world population requires food security, which can be partly achieved by reducing the portion of food lost every year as a result of pests (Kumar and Omkar, 2018). However, yield losses inflicted by crop pests have been observed to increase constantly despite different strategies being implemented globally (Dhaliwal *et al*., 2010).

Tomato (*Solanum lycopersicum* L.) is one of the most popular vegetables in the world and its fruits are a rich source of nutrients and health-promoting compounds (Luna-Guevara *et al*., 2014; Asensio *et al*., 2019). One average-sized tomato fruit offers 40% and 20% of the recommended daily amount of vitamins C and A, respectively. It also provides a significant amount of dietary fibres and minerals like calcium and potassium (Tigist *et al*., 2011). Furthermore, the antioxidant activity of ascorbic acid, carotenoids, and phenols protects humans against cancers and cardiovascular diseases (Tigist *et al*., 2011; Luna-Guevara *et al*., 2014). Therefore, any technology used on tomato crop has to be investigated not only for its effect on growth and yield, but also on fruit quality parameters.

Several pests have been reported to attack tomato throughout its production cycle (Kumar and Omkar, 2018). The tomato leaf miner, *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae), was recognised among the major pests since 1964 in Argentina from where it invaded the rest of South America (Desneux *et al*., 2010). In Rwanda, *T. absoluta* was first recorded in Bugesera District in 2015 (FAO, 2015), after which it quickly spread in all tomato production areas of the country. The damage inflicted by *T. absoluta* affects negatively its growth and development and can lead to total crop failure (Biondi *et al*., 2018). This calls for concerted efforts from different stakeholders in developing effective management strategies for this devastating pest.

Synthetic pesticides have been observed to be less effective against *T. absoluta* (Roditakis *et al*., 2013) and are associated with various challenges and harmful effects (Braham *et al*., 2012; Kumar and Omkar, 2018). The concept of integrated pest management (IPM) was developed to address the drawbacks of solely relying on chemical control. In this perspective, alternatives to chemical control with reduced negative effects have been the object of research in several parts of the world (Biondi *et al*., 2018). A lot has been done on natural enemies and biopesticides, which are used in biological control of *T. absoluta* in some parts of the world (Desneux *et al*., 2010; El-Ghany *et al*., 2016; Giorgini *et al*., 2019).

However, no study has been conducted in Rwanda to evaluate different *T. absoluta* management options for their effect on growth, yield and fruit quality of tomato.

Entomopathogenic nematodes (EPNs), entomopathogenic fungi (EPFs) and plant extracts (PE) are among the claimed options for effective management of *T. absoluta* (Mansour *et al*., 2018; Mantzoukas and Eliopoulos, 2020). Laboratory studies recommended some EPNs, EPFs, and PEs, which can be advanced to field evaluation stage. The current study investigated the growth, yield and fruit quality of tomato as affected by entomopathogens and plant extracts against *T. absoluta*.

7.2 Materials and methods

7.2.1 Study site

This study was carried out in Bugesera district of Rwanda, in a farmer's field located at 02^{o} 32' 355" South latitude, 030^{o} 26' 963" East longitude and an elevation of 1338 m above sea level. The average annual rainfall and temperature are 854 mm and 21.4^{o}C, respectively (Kabirigi *et al.,* 2017).

7.2.2 Experimental design, trial establishment, and treatment application

The study evaluated nine treatments in a randomised complete block design with three replications. The individual experimental plots were 3 m long and 2 m wide, with 1.5 m wide paths between them. Thirty days old, healthy and uniform tomato seedlings were transplanted into the plots applied with 20 t of organic manure per hectare and mulched with dry grass. Transplanting for trials one and two was carried out on 3rd April 2019 and 28th June 2019, respectively.

The treatments included: two local EPN isolates (*Steinernema* sp. RW14-M-C2a-3 and *Steinernema* sp. RW14-M-C2b-1), two commercial formulations of EPFs [Metatech® WP: *Metarhizium anisopliae* (Metsch.) Sorok, Strain FCM Ar 23B3, 5 x 10^{9} CFUs/g, and Beauvitech® WP: *Beauveria bassiana* (Bals.) Vuill., Strain J25, 1 x 10^{10} CFUs/g], two local plant extracts (*Tephrosia vogelii* and *Phytolacca dodecandra*), azadirachtin 0.03% EC (Nimbecidine), imidacloprid (Confidor SL 200) and water. The two last treatments were included as positive and negative controls, respectively. The two EPN isolates used were obtained from Biological Control Laboratory – EPN Production Facility at Rwanda Agriculture and Animal Resources Development Board (RAB) (Yan *et al*., 2016).

Mass production of the EPNs was done through *in-vivo* method using *Galleria mellonella* larvae (Kaya and Stock, 1997). For field applications, these EPNs were formulated into sponges and were used at a concentration of 5 x 10^9 IJs/ha **(**Gözel and Kasap, 2015).

The EPF formulations were obtained from Dudutech Division, Flamingo Horticulture (K) Ltd, Naivasha, Kenya and were used at a concentration of 250 g/ha. The two local plant extracts were prepared from leaves of local plants (*T. vogelii* and *P. dodecandra*). The fine powder was obtained (using an electric grinder) from the leaves dried in a shaded area, mixed with boiled water and kept for 12 h. The concentration used for field application was 15% weight/volume (w/v) and filtration was done using a muslin cloth. Azadirachtin 0.03% EC (Nimbecidine) and imidacloprid (Confidor SL 200) were used at the rates of 5 and 1 mL, respectively, per litre of water. All these treatments were applied weekly using a knapsack sprayer and the application volume was 1000 L/ha (Brusselman *et al*., 2012).

7.2.3 Cultural operations

Apart from the difference in applied treatments, all other cultural operations were uniformly done in all the experimental plots. Fungicide application was done every week by alternating Copper oxychloride 50% WP with fungicides containing Mancozeb 80% or Mancozeb (640 g/kg) + Metalaxyl (80 g/kg). Each tomato plant was fertilised with 10 g of NPK 17-17-17 as basal fertiliser, supplemented with 4 g of Urea 46% on 30th day after transplanting as per RAB recommendation. Other cultural practices like watering, weeding, and pruning were carried out conventionally.

7.2.4 Data collection and analysis

Data were collected on growth, yield and fruit quality parameters. Plant growth parameters, namely plant height, stem diameter, and number of leaves per plant, were recorded every two weeks. Plant height (cm) was measured from the ground to the tip of each plant using a metre tape. Stem diameter (mm) was measured from the collar using a digital vernier calliper. The number of leaves arising from the main stem was counted. For yield parameters, the numbers of flower trusses per plant and flowers per truss were recorded 40 days after transplanting, while the number of fruits per truss was recorded 60 days after transplanting. The numbers and yield of healthy and bored fruits were recorded during the harvesting period, which started 72 and 70 days after transplanting in trials one and two, respectively. All the above parameters were taken from five plants selected randomly in the middle of each plot.

Fruit quality parameters, namely: fruit firmness (kgF/cm^2), total soluble solids (TSS) (^{0}Brix), beta-carotene (mg/100 g of fruit), lycopene (mg/100 g of fruit), and ascorbic acid (mg/100 g of fruit), were recorded. To determine fruit firmness, tomatoes were harvested at the pink stage and stored at room temperature until the uniform red ripe stage. Then, five fruits were randomly selected from each treatment lot and fruit firmness measured in the equatorial zone of each tomato using a penetrometer (Ritenour *et al*., 2002). Total soluble solids were determined on the same fruits used for the determination of fruit firmness using a refractometer (RHW Refractometer, Optoelectronic Technology Company Limited, UK) (Majidi *et al*., 2011). Beta-Carotene was obtained following the method described by Delia *et al*. (2004). Lycopene was extracted using acetone and analysed in a spectrophotometer at 503 nm. Lycopene content was then calculated using the formula given by Ranganna (1997) as follows:

$$Lycopene\ content = 3.1206\ x\ A\ x\ V\ x\ D\ x \frac{100}{W\ x\ 100}$$

where A = Absorption, V = Volume made up, D = Dilution, W = Weight of Sample.

Ascorbic acid was determined by titration with 2,6-dichlorophenolindophenol dye (AOAC, 1990).

The distribution of the collected data was assessed and the appropriate transformation was undertaken, where necessary, before subjecting them to analysis of variance. In both trials, the numbers of healthy and bored fruits per plant were square-root transformed, while the yield of healthy and bored fruits per plant was log-transformed. The number of fruits per truss was log-transformed in trial one, and arcsine-transformed in trial two; while the number of flowers per truss was arcsine-transformed in trial two. All other parameters were analysed without transformation. To determine the effects of the treatments on growth, yield and fruits quality of tomato, analysis of variance was carried out; and the means for significantly different treatments (at $p \leq 0.05$) were separated using Tukey's honestly significant difference test. The data analysis was carried out using the Statistical Analysis System package, SAS software version 9.2 (SAS Institute, 2010). The statistical model fitted for this experiment was the same as indicated in section 6.2.5.

7.3 Results

7.3.1 Effect of entomopathogens and plant extracts on tomato growth

Plant height

The studied treatments significantly ($p \leq 0.05$) influenced tomato plant height from 30 days after transplanting (DAT) (Figure 7.1). In both trials, the plant height was not significantly different at 15 DAT; with an average of 14.9 and 15.3 cm for trials one and two, respectively. Plant height increased with time but became almost constant at 45 DAT. In trial one, there was no significant difference among the entomopathogens (EPNs and EPFs) and azadirachtin on all days of observation. *Tephrosia vogelii* was not significantly different from all the above at 30, 45, 60 DAT, except *Steinernema* sp. RW14-M-C2a-3. Lower plant height was recorded with *P. dodecandra* and the controls which were not significantly different. In trial two, plant height did not differ significantly among the treatments, except *P. dodecandra* and the controls, which had lower plant height than others.

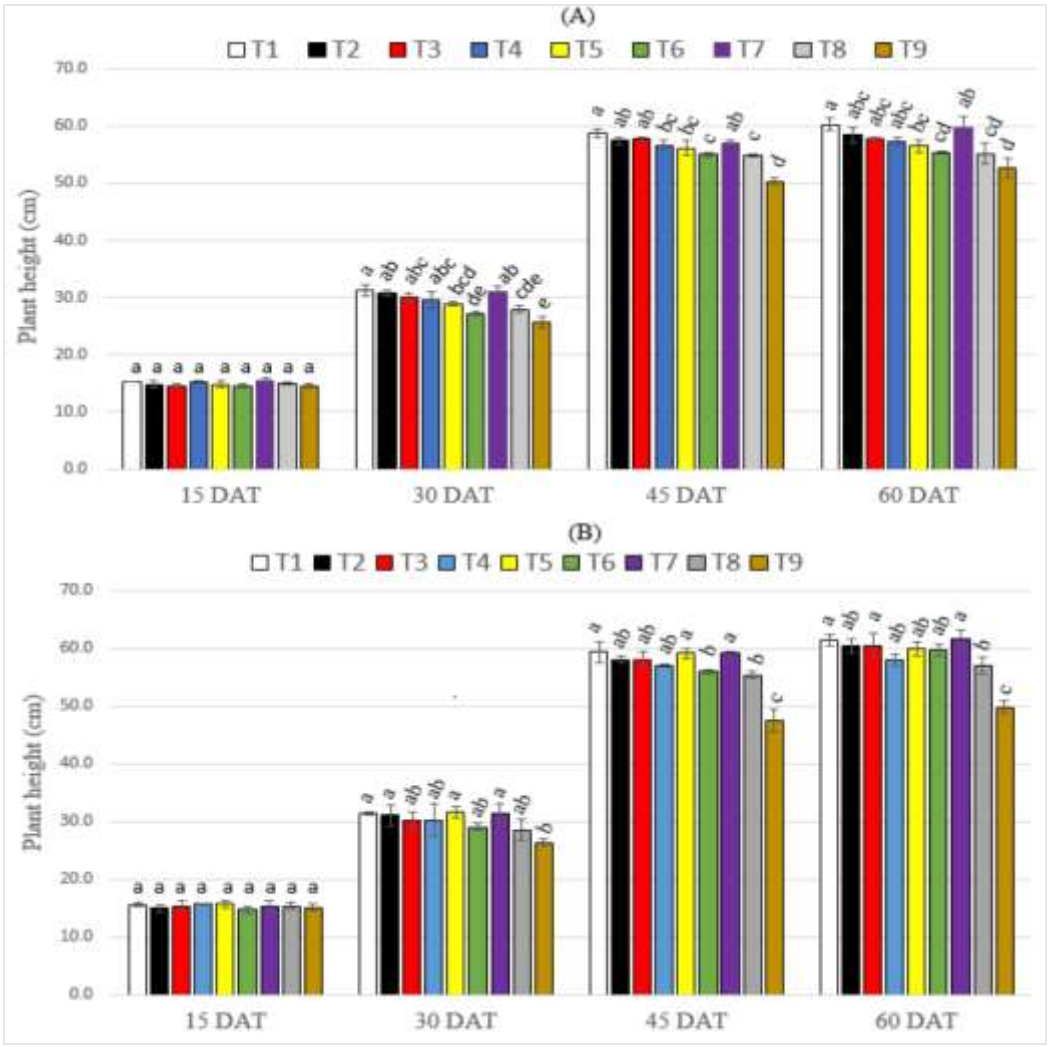

Figure 7.1 Height of tomato cv. Roma under different treatments against *Tuta absoluta* in trials one (A) and two (B). T1: *Steinernema* sp. RW14-M-C2a-3, T2: *Steinernema* sp. RW14-M-C2b-1, T3: Metatech® WP (*Metarhizium anisopliae*, Strain FCM Ar 23B3), T4: Beauvitech® WP (*Beauveria bassiana*, Strain J25), T5: *Tephrosia vogelii, T6: Phytolacca dodecandra*, T7: Azadirachtin 0.03% EC, T8: Imidacloprid, T9: Water; DAT: Days after transplanting; Different letters above the bars indicate significant difference according to Tukey's test ($p \leq 0.05$).

Stem diameter

Treatments did not differ in stem diameter at 15 and 30 days after transplanting (DAT) in trial one and at 15 DAT in trial two. In addition, only the stem diameter in negative control was significantly lower as compared to the other treatments at 45 DAT in trial one. Imidacloprid and *P. dodecandra* were similar to the negative control, with significantly lower stem diameter ($p \leq 0.05$) as compared to the other treatments at 60 DAT (Figure 7.2).

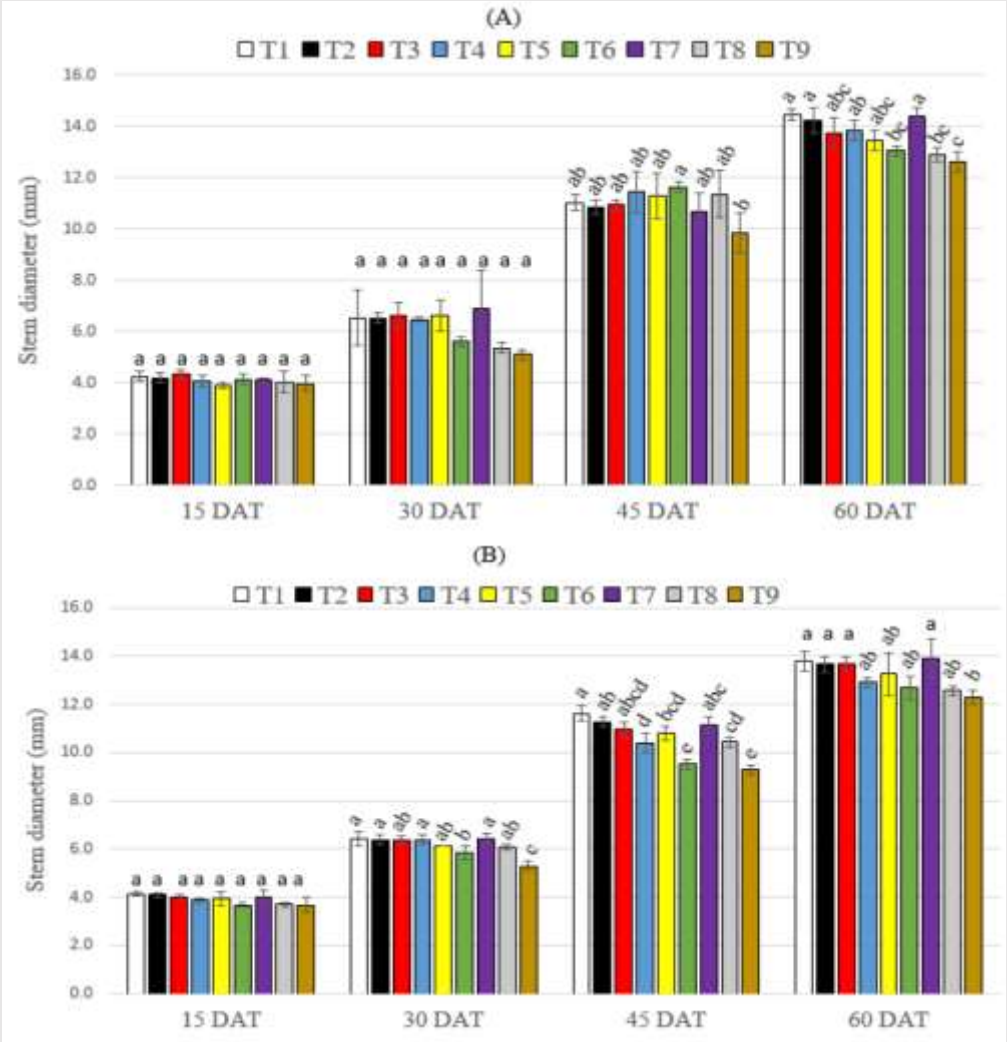

Figure 7.2 Stem diameter of tomato cv. Roma under different treatment against *Tuta absoluta* in trials one (A) and two (B). T1: *Steinernema* sp. RW14-M-C2a-3, T2: *Steinernema* sp. RW14-M-C2b-1, T3: Metatech® WP (*Metarhizium anisopliae*, Strain FCM Ar 23B3), T4: Beauvitech® WP (*Beauveria bassiana*, Strain J25), T5: *Tephrosia vogelii, T6: Phytolacca dodecandra*, T7: Azadirachtin 0.03% EC, T8: Imidacloprid, T9: Water; Different letters above the bars indicate significant difference according to Tukey's test ($p \leq 0.05$).

For trial two, *P. dodecandra* and negative control had significantly lower stem diameter as compared to the other treatments at 30 DAT; but at 60 DAT it was only the negative control, which had significantly lower stem diameter as compared to azadirachtin and all entomopathogens except Beauvitech® WP (T4).

Number of leaves per plant

The evaluated treatments did not significantly affect the number of leaves per plant in both trials (Figure 7.3).

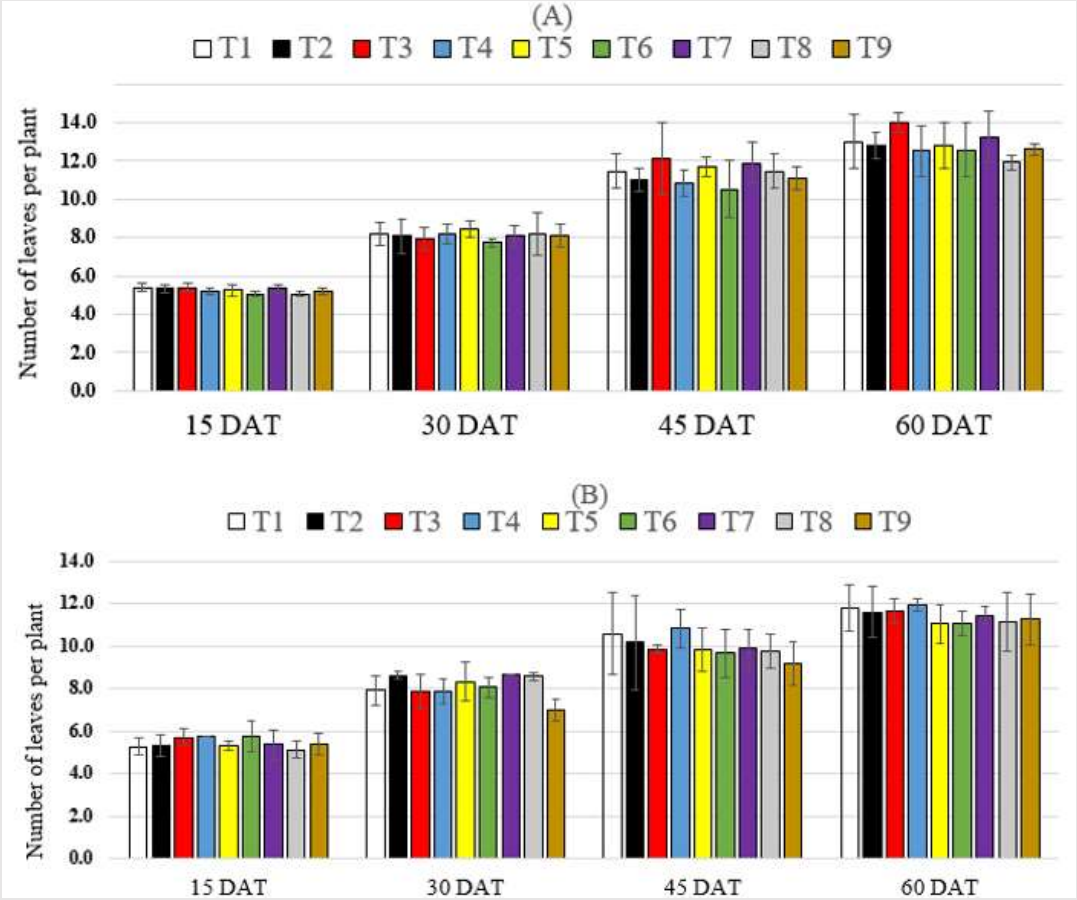

Figure 7.3 Number of leaves per plant for tomato cv. Roma under different treatment against *Tuta absoluta* in Trials one (A) and two (B).T1: *Steinernema* sp RW14-M-C2a-3, T2: *Steinernema* sp. RW14-M-C2b-1, T3: Metatech® WP (*Metarhizium anisopliae*, Strain FCM Ar 23B3), T4: Beauvitech® WP (*Beauveria bassiana*, Strain J25), T5: *Tephrosia vogelii, T6: Phytolacca dodecandra*, T7: Azadirachtin 0.03% EC, T8: Imidacloprid, T9: Water.

However, the general trend observed in both trials was that slightly higher (but not significantly different) number of leaves per plant could be obtained in plots treated with Metatech® WP (*M. anisopliae*, Strain FCM Ar 23B3) and azadirachtin in trial one; and with *Steinernema* sp RW14-M-C2a-3 and Beauvitech® WP (*B. bassiana*, Strain J25) in trial two (Figure 7.3). In trial one, the average number of leaves per plant was 5.3, 8.1, 11.4 and 12.8; while in trial two, it was 5.5, 81, 10.0 and 11.4 at 15, 30, 45 and 60 days after transplanting, respectively.

7.3.2 Effect of entomopathogens and plant extracts on tomato yield

The evaluated treatments significantly ($p < 0.001$) influenced tomato yield parameters in both trials. Generally, plots treated with the entomopathogens or azadirachtin had a higher performance as compared to those with plant extracts or controls. A similar number of flower trusses per plant was recorded by *Steinernema* sp. RW14-M-C2a-3, *Steinernema* sp. RW14-M-C2a-3, Metatech® WP, Beauvitech WP, and azadirachtin. These values were significantly ($p < 0.001$) higher than *T. vogelii*, *P. dodecandra*, imidacloprid and water spray in trial one. In trial two, the effect of *T. vogelii* and imidacloprid was similar to all the treatments, except the plots treated with EPNs and azadirachtin, which recorded a significantly higher number of flower trusses per plant. The number of flowers per truss was significantly higher with *Steinernema* sp. RW14-M-C2a-3 and azadirachtin in trial one, and with all entomopathogenic nematodes and azadirachtin in trial two. Higher numbers of fruits per truss, healthy and bored fruits per plant were recorded with all entomopathogens and azadirachtin, in both trials. A similar trend was observed in the yield of healthy and bored fruits per plant (Table 7.1).

Table 7.1 Yield parameters (mean ± SD) of tomato under different entomopathogens and plant extracts treatments

Treatments	Number of flower trusses/plant	Number of flowers/truss	Number of fruits/truss	Number of healthy fruits/plant	Number of bored fruits/plant	Yield of healthy fruits (g / plant)	Yield of bored fruits (g/plant)
TRIAL ONE							
T1	11.9 ± 0.2 a*	9.7 ± 0.4 a	4.1 ± 0.3 a	5.9 ± 0.2 a	5.8 ± 0.2 a	406.3 ± 10.9 a	333.3 ± 33.4 a
T2	11.6 ± 0.4 abc	8.5 ± 0.1 bc	4.0 ± 0.1 a	5.6 ± 0.2 a	5.1 ± 0.4 a	381.0 ± 22.3 a	286.8 ± 8.8 a
T3	11.7 ± 0.2 ab	7.7 ± 0.2 cd	4.1 ± 0.1 a	5.5 ± 0.3 a	6.1 ± 0.4 a	374.4 ± 23.5 a	328.8 ± 34.9 a
T4	11.4 ± 0.1 abc	7.3 ± 0.3 de	3.8 ± 0.5 a	5.4 ± 0.2 a	5.6 ± 0.4 a	335.0 ± 34.5 a	313.9 ± 17.3 a
T5	10.9 ± 0.2 bc	7.4 ± 0.1 de	3.0 ± 0.3 b	2.5 ± 0.3 b	3.2 ± 0.2 b	151.0 ± 12.3 b	161.7 ± 9.7 b
T6	10.8 ± 0.3 c	6.4 ± 0.2 ef	2.9 ± 0.2 b	1.5 ± 0.1 c	2.6 ± 0.2 cb	81.5 ± 12.5 b	126.2 ± 10.1 b
T7	12.7 ± 0.1 a	9.4 ± 0.6 ab	4.3 ± 0.2 a	6.5 ± 0.2 a	4.9 ± 0.6 a	402.9 ± 12.7 a	275.7 ± 29.5 a
T8	10.8 ± 0.3 c	6.5 ± 0.3 ef	3.0 ± 0.4 b	1.7 ± 0.2 c	2.3 ± 0.5 cb	86.6 ± 9.0 b	109.9 ± 26.2 bc
T9	10.8 ± 0.5 c	5.6 ± 0.7 f	2.5 ± 0.3 b	0.7 ± 0.4 d	1.9 ± 0.5 c	32.5 ± 8.2 c	83.7 ± 22.1 2 b
CV	2.50	4.89	6.53	4.39	5.76	4.20	2.23
P	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
TRIAL TWO							
T1	9.0 ± 0.2 a	7.7 ± 0.3 a	3.8 ± 0.3 a	5.5 ± 0.2 a	4.6 ± 0.7 a	367.8 ± 5.2 a	249.5 ± 22.1 a
T2	9.0 ± 0.2 a	7.4 ± 0.2 a	3.7 ± 0.2 a	5.4 ± 0.2 a	4.7 ± 1.0 a	350.9 ± 12.1 a	255.9 ± 38.1 a
T3	8.8 ± 0.2 ab	5.6 ± 0.2 cb	3.8 ± 0.4 a	4.9 ± 0.4 a	5.6 ± 0.8 a	309.7 ± 26.3 a	302.4 ± 45.9 a
T4	8.8 ± 0.5 ab	5.5 ± 0.2 cb	3.6 ± 0.2 a	4.9 ± 0.4 a	5.3 ± 1.0 a	319.4 ± 33.5 a	273.6 ± 64.8 a
T5	8.8 ± 0.2 ab	5.9 ± 0.2 b	2.8 ± 0.1 b	2.0 ± 0.4 b	4.4 ± 0.4 a	113.1 ± 13.4 b	220.9 ± 18.2 a
T6	8.1 ± 0.2 b	5.2 ± 0.3 cb	2.4 ± 0.4 b	1.4 ± 0.2 b	2.1 ± 0.1 b	67.0 ± 8.5 c	114.8 ± 17.0 b
T7	9.3 ± 0.2 a	8.0 ± 0.3 a	4.0 ± 0.2 a	6.0 ± 0.4 a	4.7 ± 0.1 a	392.5 ± 38.1 a	266.6 ± 19.6 a
T8	8.7 ± 0.1 ab	5.5 ± 0.3 cb	2.7 ± 0.2 b	1.6 ± 0.3 b	1.9 ± 0.5 b	74.7 ± 8.1 c	96.7 ± 22.5 b
T9	8.1 ± 0.2 b	5.0 ± 0.2 c	2.2 ± 0.3 b	0.8 ± 0.2 c	1.7 ± 0.2 b	35.7 ± 6.7 d	80.0 ± 10.10 b
CV	3.02	2.31	4.17	5.11	7.94	2.30	3.05
P	0.0004	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001

T1: *Steinernema* sp. RW14-M-C2a-3, T2: *Steinernema* sp. RW14-M-C2b-1, T3: Metatech® WP (*Metarhizium anisopliae*, Strain FCM Ar 23B3), T4: Beauvitech® WP (*Beauveria bassiana*, Strain J25), T5: *Tephrosia vogelii, T6: Phytolacca dodecandra*, T7: Azadirachtin 0.03% EC, T8: Imidacloprid, T9: Water. Means followed by the same letter (s) are not significantly different (Tukey's test, $p \leq 0.05$)

7.3.3 Effect of entomopathogens and plant extracts on tomato fruit quality

Tomato fruit quality parameters were not significantly influenced by the applied treatments against *T. absoluta*. The results obtained were so close to each other that it is not easy to find any trend amongst the treatments. The average values obtained were 3.2 and 3.3 kgF/cm^2 for fruit firmness, 4.2 and 4.4 ^{o}Brix for TSS, 8.3 and 8.1 mg/100 g of fruit for beta-carotene, 5.4 and 5.5 mg/100 g of fruit for lycopene, 14.36 and 14.6 mg/100 g of fruit for ascorbic acid, in trials one and two, respectively (Figure 7.4).

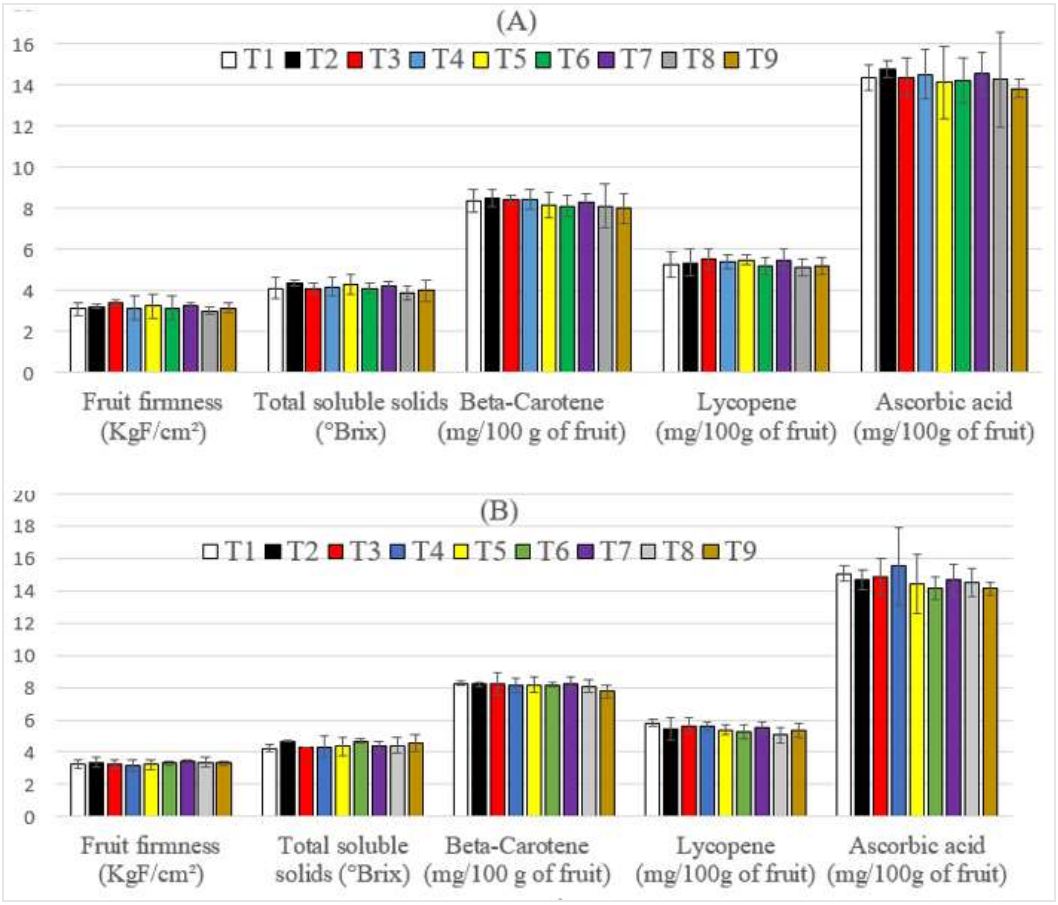

Figure 7.4 Quality parameters of tomatoes cv. Roma (mean ± SD) under different treatments of entomopathogens and plant extracts against *Tuta absoluta* in Trials one (A) and two (B). T1: *Steinernema* sp RW14-M-C2a-3, T2: *Steinernema* sp. RW14-M-C2b-1, T3: Metatech® WP (*Metarhizium anisopliae*, Strain FCM Ar 23B3), T4: Beauvitech® WP (*Beauveria bassiana*, Strain J25), T5: *Tephrosia vogelii*, *T6: Phytolacca dodecandra*, T7: Azadirachtin 0.03% EC, T8: Imidacloprid, T9: Water.

7.4 Discussion

The effects of entomopathogens and plant extracts on growth, yield and fruit quality of tomato have never been investigated in Rwanda. The significant differences observed in plant height and stem diameter could be due to the differences in the efficacy of studied treatments against *T. absoluta*. The damages inflicted by *T. absoluta* larvae may have affected the physiological and biochemical reactions of tomato plants, so that plant growth was consequently affected (Desneux *et al*., 2010). *Beauveria bassiana*, which was reported to exhibit endophytic activity by colonising vascular tissues (Nishi *et al*., 2020), would be expected to impair the normal plant growth. However, different researchers reported that *B. bassiana* does not impede tomato growth (Klieber and Reineke, 2016; Allegrucci *et al*., 2017). On the other hand, since *T. vogelii* is a rich source of nitrogen, through biological nitrogen fixation (Stevenson *et al*., 2012), more growth would be expected in this treatment as compared to other treatments because nitrogen is more involved in plant growth and biomass production (Larbat *et al*., 2016). This was, however, not observed in this study and could be explained by the fact that the amount sprayed as an insecticide was too little to have a direct significant effect on plant growth. Finally, the non-significant difference in the number of leaves per plant despite the treatments could be because this parameter is associated with the genetic makeup of the plant (Kaushik *et al*., 2011) and not with cultural practices including pest management.

The significant difference in flower-related parameters could also be due to the difference in the efficacy of the studied treatments. By attacking the floral parts, *T. absoluta* larvae might have damaged some of them before they differentiate into flowers and caused others to drop; which could be the explanation for the flower abortion observed in this study. These results are in agreement with Cherif *et al*. (2013) who reported that *T. absoluta* larvae can damage tomato flower parts and cause flower drop.

The observed significant difference in yield parameters may also have arisen from the indirect effect of *T. absoluta* larvae through their feeding activity in leaf mesophyll (Biondi *et al.*, 2018), which might have slowed down the process of assimilates synthesis and partitioning for their utilisation by different plant organs, including flower parts and fruits. In agreement with the above observation, Desneux *et al*. (2010) and El-Ghany *et al.* (2016) also reported that tomato attack by *T. absoluta* disturbs its normal growth, development and the subsequent yield. Thus, higher numbers of flower trusses per plant, flowers per truss, and

fruits per truss recorded with entomopathogens and azadirachtin suggest that these treatments can reduce tomato yield loss as compared to the plant extracts and the controls (imidacloprid and water spray).

In their study, Rab and Haq (2012) found that the number of flowers per truss varied from 17.13 to 30.77 while the number of fruits per cluster was 4.05 to 6.35 for tomato cv. Roma. However, in the present study, a range of 5.6 - 9.7 flowers per truss and 2.2 - 4.3 fruits per truss was obtained. This indicates the ability of *T. absoluta* to affect negatively the flower and fruit-bearing capacity of a tomato plant. This is one of the reasons for high yield losses frequently observed with *T. absoluta* infestations (Cherif *et al*., 2013; Biondi *et al*., 2018).

The higher number and yield of healthy fruits that were obtained with EPNs, EPFs, and azadirachtin, support our earlier findings in laboratory experiments (Chapters three, four and five). In line with the findings of this study, Braham *et al*. (2012), Gözel and Kasap (2015), Yousef *et al*. (2015), and El-Ghany *et al.* (2016) reported that EPNs, EPFs, and azadirachtin result in better control of *T. absoluta*. The performance of plant extracts and imidacloprid (positive control) remained low as it was in the laboratory studies. Negative control also recorded very low yield, which was consistent with Desneux *et al*. (2011) and Biondi *et al*. (2018) who emphasized that if there are no serious pest management strategies that are meticulously implemented, the yield loss might reach 100%.

Higher number and yield of bored fruits obtained from plots treated with entomopathogens and plant extracts, as compared to plant extracts and controls, might have resulted from the reduced number of aborted and damaged flowers by *T. absoluta* in the plots where these treatments were applied. However, even though these fruits survived from early abortion and the dropping of progenitor flowers, they were more exposed to *T. absoluta* because they were many, and thus a group of them was bored later by the pest that spoiled their quality.

Compared to the negative control, the yield of healthy fruits obtained with *Steinernema* sp. RW14-M-C2a-3, *Steinernema* sp. RW14-M-C2a-3, Metatech® WP, Beauvitech® WP, and azadirachtin increased 12.5, 11.7, 11.5, 10.3, and 12.4 folds, respectively. While compared to the positive control, it was 4.8, 4.5, 4.2, 4.1 and 5.0 folds, respectively. This confirms that, despite the invasive nature of *T. absoluta*, different management options can reduce significantly its negative impact on the crop. However, dependence on chemical pesticides should be discouraged as evidenced by the results of this study, which are consistent with several other researchers (Desneux *et al*., 2010; Roditakis *et al*., 2013; Biondi *et al*., 2018).

The commercial value of bored fruits is lost because they are not preferred by customers as external appearance and absence of defects are among the factors determining consumer preference (Asensio *et al.,* 2019). In addition to the larvae that enter inside the fruits, also some pathogens like fungi often get inside through the created holes and cause fruit decaying before or after harvest (Desneux *et al*., 2010). The findings of this study are supported by previous researchers who worked on other pests and reported that crop pests are among the main factors reducing the yield and quality of field horticultural produce by direct feeding or by favouring several diseases (Kumar and Omkar, 2018). Thus, the implementation of IPM is worth to ensure better yield and quality of tomato crop.

In this study, non-significant difference observed in fruit quality parameters suggests that the studied treatments had no direct effect on these parameters. According to Marsic *et al*. (2011), Rab and Haq (2012), Tigist *et al*. (2011) and Asensio *et al.* (2019), tomato fruit quality is determined by factors such as variety, plant nutrition, and climatic conditions, among others. For instance, Tigist *et al*. (2011) observed from their study that fruit firmness, TSS and ascorbic acid varied significantly among different tomato varieties. Rab and Haq (2012) found that foliar application of calcium chloride and borax influences the quality of tomato fruits. These findings are consistent with the results of the present study where variety, plant nutrition, and climatic conditions were not varied; which may be the cause of the lack of significant difference.

Fruit firmness results obtained in this study fall in the range of the values obtained by Rab and Haq (2012). Fruit firmness is an important quality parameter that determines fruit shelf-life and resistance to mechanical damage (Tigist *et al*., 2011). In line with the current study, Parmar *et al.* (2018) also obtained a TSS value of 4.8 ^{o}Brix for tomato cv. Roma under organic management system. Also, TSS values obtained by Rab and Haq (2012) ranged from 4.08 to 6.10 ^{o}Brix under different rates of calcium chloride and borax. The values of beta-carotene and lycopene recorded in this study are close to what was obtained by Parmar *et al.* (2018) (8.34 and 5.38 mg/100 g of fruit, respectively) for the same variety (Roma) produced organically. The ascorbic acid results obtained in this study agree with the earlier findings of Tigist *et al*. (2011) who obtained values of 13.2 and 14.8 mg/100 g after four and eight days of room temperature storage, respectively, for Tomato cv. Roma fruits harvested at green mature stage.

According to Tigist *et al*. (2011), these quality parameters develop into fruit during the pre-harvest period and they do not get improved after harvesting. However, they can be maintained by proper post-harvest handling and storage. Since pre-harvest activities are responsible for the development of quality parameters in tomato fruits, any technology used to improve its production should also be assessed for its effect on fruit quality.

7.5 Conclusion

The studied entomopathogens and plant extracts significantly affected tomato growth and yield but not the fruit quality parameters. Better yield performance can be obtained with the entomopathogenic nematode isolates (*Steinernema* sp. RW14-M-C2a-3 and *Steinernema* sp. RW14-M-C2a-3), commercial formulations of entomopathogenic fungi (Metatech® WP: *Metarhizium anisopliae*, Strain FCM Ar 23B3 and Beauvitech® WP: *Beauveria bassiana*, Strain J25) and azadirachtin 0.03% EC, which were not significantly different. These biorational control agents are recommended to be included in the IPM of *Tuta absoluta* in Rwanda. The results of this study will guide producers to select the best control options that can result in higher comparative growth and yield without compromising fruit quality.

CHAPTER EIGHT

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

8.1 General Discussion

Since the tomato leaf miner, *Tuta absoluta*, has become a major threat to tomato production in Rwanda (Uzayisenga *et al*., 2016), urgent actions are needed to deal with this invasive pest, which has scared many farmers and even forced some to abandon tomato production (Zekeya *et al*., 2017). Furthermore, looking at the experience from South America and Europe where overuse of chemical pesticides resulted in a build-up of resistant populations of *T. absoluta* (Mansour *et al*., 2018), action is needed to avoid the same situation in Rwanda. The development of options for IPM of *T. absoluta* was the main focus of this study.

This work started with a series of laboratory experiments to identify the biorational agents which can control *T. absoluta.* The potential of using local EPN isolates (Yan *et al*., 2016) was assessed (Chapter three) and it was observed that all evaluated local isolates caused higher *T. absoluta* larval mortality even from 24 h after inoculation. The two outstanding isolates, *Steinernema* sp. RW14-M-C2a-3 and *Steinernema* sp. RW14-M-C2b-1 were recommended to be advanced to the field evaluation stage. The existence of an EPNs production factory in Rwanda (Holmes *et al*., 2015) complements efforts to obtain the most effective EPN isolates, which could be mass multiplied and used in IPM for *T. absoluta*. Being indigenous and locally adapted to Rwandan conditions, these EPNs would be more effective in local field conditions as compared to exotics which would need more time to adapt to a new environment (Lima *et al*., 2017).

The pathogenicity of commercial formulations of EPFs was also evaluated (Chapter four) where two formulations, Metatech® WP (*M. anisopliae*, Strain FCM Ar 23B3) and Beauvitech® WP (*B. bassiana*, Strain J25) exhibited higher pathogenicity (lower LT_{50} values) even though the EPFs took long to kill their hosts (4 – 6 days) as compared to the EPNs. The faster rate of host-killing for EPNs could be linked mainly to the associated symbiotic bacteria which help them to invade and overcome the immune system of their hosts in a short time (Stock, 2015). One of the challenges in adopting EPFs for controlling insect pests is this behaviour of killing their host somehow slowly. However, some scientists are eager to find ways of reducing the time required to kill 50% (LT_{50}) or 90% (LT_{90}) of the pest population using EPFs. For instance, Wang and St Leger (2007) were able to genetically

modify *M. anisopliae* so that it expresses a neurotoxin from the scorpion *Androctonus australis* and this significantly reduced LT_{50} and LT_{90}. In the same way, Boldo *et al.* (2009) obtained more than 20% reduction in the LT_{50} and LT_{90} against the Peruvian larder beetle (*Dermetes peruvianus*) and thus increased virulence by overexpressing the CHI2 chitinase of *M. anisopliae*. Similarly, Borgi *et al*. (2016) evaluated a spontaneous mutant of *B. bassiana,* P2 that was selected from a local Tunisian strain P1, and found that P2 expressed more virulence against *T. absoluta* due to higher production of proteases and chitinases known to hydrolyse cell wall. P2 resulted in LC_{50} values which were 10 times lower than those of P1 and caused insect mortality up to 99% within five days. This emphasizes the need for continuous evaluation of different strains as their virulence toward the pest can be very different.

Other studies also reported higher laboratory efficacy of EPNs as compared to EPFs (Youssef, 2015). While some studies reported that EPFs can attack all *T. absoluta* development stages (Schrank and Vainstein, 2010), low efficacy of EPNs was reported against pupae (Batalla-Carrera *et al*., 2010; Garcia-del-Pino *et al*., 2013). In addition, Klieber and Reineke (2016) reported that EPFs can slow down the feeding activity of insects as they die gradually. Thus, long term effect will be expected with the use of EPFs, because the EPFs were reported to significantly reduce the pest population. Furthermore, the endophytic behaviour of *B. bassiana (*Allegrucci *et al*., 2017; Nishi *et al*., 2020) enables its effective distribution in different parts of the plants to access the larvae in galleries.

The selected plant extracts were also evaluated for their bioactivity against *T. absoluta* (Chapter five) where lower mortality rates were obtained, as compared to the EPNs and EPFs. The highest mortality rates recorded five days after treatment were 35.1% and 24.9% for *T. vogelii* and *P. dodecandra*, respectively while azadirachtin had caused more than 64% mortality after only two days and 100% after 5 days. This low efficacy of plant extracts could be due to the concentration (10% w/v) used and the extraction method where water was used as a solvent. However, even in field conditions, with a concentration of 15% w/v, their efficacy remained low. *In-vitro* efficacy of azadirachtin was comparatively lower than the two selected EPNs, but it was higher than the EPFs. In contrast, El-Ghany *et al*. (2016) obtained higher laboratory efficacy of *B. bassiana* compared to azadirachtin, which implies that the efficacy of entomopathogen may be strain-dependent. Due to the higher efficacy, azadirachtin was recommended to be advanced to the field evaluation stage of this study.

In field conditions, the selected EPNs (*Steinernema* sp. RW14-M-C2a-3 and *Steinernema* sp. RW14-M-C2b-1), EPFs (Metatech® WP: *M. anisopliae*, Strain FCM Ar 23B3 and Beauvitech® WP: *B. bassiana*, Strain J25), plant extracts (*T. vogelii* and *P. dodecandra*), and azadirachtin were evaluated for their efficacy and effects on growth, yield, and fruit quality of tomato. The entomopathogens and azadirachtin exhibited higher performance in terms of leaf and leaflet damage, plant height, stem diameter, and yield parameters as compared to plant extracts and the controls (imidacloprid and water). The number of leaves per plant and fruit quality parameters were not significantly affected by the treatments. The treatments which had lower leaf and leaflet damages resulted, generally, in plants with higher plant height and stem diameter. In line with this, different researchers reported that the more the *T. absoluta* infestation, the lower the overall crop performance (Desneux *et al*., 2010; Biondi *et al*., 2018). El-Ghany *et al.* (2018) reported that *T. absoluta* damages on tomato crop affect negatively its photosynthesis, growth, and yield. Thus, the lower performance obtained with plant extracts and the controls could be attributed to the negative effects on the physiology of stressed plants including the production of carbohydrates through photosynthesis.

The entomopathogens and azadirachtin, which significantly reduced leaf and leaflet damages ($p < 0.05$), as compared to the plant extracts and controls, also reduced yield losses. These findings are in agreement with Cocco *et al*. (2014) who reported the existence of a positive correlation between leaf and fruit infestations by larvae of *T. absoluta*. Previous studies also indicated that *T. absoluta* larvae concentrate primarily on leaves and infest fruits later after their population has exceeded a certain density (Braham *et al*, 2012; Cherif *et al*., 2013; Cocco *et al*., 2014). Correspondingly, Cocco *et al*. (2014) observed in a greenhouse that the leaf infestation rates of 36%, 43% and 60% for tomato varieties with big, medium and small-sized fruits, respectively, are associated with about 1% damaged fruits.

Different studies revealed that the combined use of different pest management options may result in improved efficacy than if each option was used alone (Mahmoud, 2016). Other researchers emphasized that *T. absoluta* management would be achieved by combining different strategies such as physical, cultural, biological, and careful use of pesticides (Gözel and Kasap, 2015; Jallow *et al*., 2019). This implies the IPM concept, which is the integration of carefully selected and compatible pest management options that are ecologically, economically and sociologically acceptable (Rao and Tanweer, 2011).

Mutegi *et al*. (2017) reported higher efficacy from a combined use of the EPN *Steinernema karii* and azadirachtin 0.03% where tomato fruit damage was 10.2% for EPN alone and 7.4% for EPN combined with azadirachtin in greenhouse conditions. This improved efficacy could be due to the fact that these two control options combined their mode of action where EPNs are efficient in killing the larvae (Batalla-Carrera *et al*., 2010) while azadirachtin acts as oviposition-deterrent, repellent, anti-feeding, as well as insect growth and development inhibitor (Senthil-Nathan, 2013). Studies have reported the compatibility of EPNs and azadirachtin, and it was found that the later does not impede the virulence and survival of the former (Gözel and Kasap, 2015).

In their study, Jallow *et al*. (2019) also achieved better results by combining azadirachtin with *B. bassiana* or with *B. thuringiensis* in greenhouse experiments; they obtained the lowest leaf damage and highest health fruit yield with these combinations as compared to each option when used individually. The higher efficacy of azadirachtin combined with *B. thuringiensi*s on *T. absoluta* was also reported by Amizadeh *et al*. (2015); while Klieber and Reneke (2016) reported similar results when azadirachtin was combined with *B. bassiana*. Tsoulnara and Port (2016) also observed higher efficacy using *B. bassiana* + *B. thuringiensis* against *T. absoluta* as compared to when they were used alone.

In the same way, Mahmoud *et al*. (2016) reported a synergistic effect between imidacloprid, Thiamethoxam, azadirachtin and *S. carpocapsae* when applied against the black cutworms, *Agrotis ipsilon*. Moreover, Patil *et al*. (2015) mentioned that the combination of imidacloprid and nematodes, *Heterorhabditis indica*, had a strong synergistic effect on mortality of early and late third instar larvae of coconut white grub. Furthermore, Ansari *et al*. (2008) observed that application of the fungus *M. anisopliae* followed by either *H. bacteriophora*, *S. feltiae* or *S. kraussei* one or two weeks later, provided 100% control of third-instar larvae of black vine weevil (*Otiorhynchus sulcatus*).

These evidences demonstrate the potential of combining EPNs or EPFs with azadirachtin, EPNs with EPFs and other different options for effective and sustainable management of *T. absoluta.* Further studies should explore this important IPM aspect to determine all possible combinations with synergic or additive effects.

8.2 Conclusions

This study aimed at contributing to enhanced tomato productivity and quality in Rwanda through evaluation of entomopathogens and plant extracts as options for integrated pest management (IPM) of tomato leaf miner (*Tuta absoluta* Meyrick). Specific objectives were formulated and to achieve them, series of laboratory bioassays, field experiments, and laboratory analyses were carried out.

From the results of this study, the following conclusions can be drawn:

i) The entomopathogenic nematode (EPN) isolates from Rwanda have the potential to control *Tuta absoluta*, with *Steinernema* sp. RW14-M-C2a-3 and *Steinernema* sp. RW14-M-C2b-1 being the most virulent. Therefore, the null hypothesis (i) is rejected.

ii) The commercial formulations of entomopathogenic fungi (EPFs) are pathogenic against *T. absoluta*, with Metatech® WP (*Metarhizium anisopliae*, Strain FCM Ar 23B3) and Beauvitech® WP (*Beauveria bassiana*, Strain J25) exhibiting higher pathogenicity. Thus, the null hypothesis (ii) is rejected.

iii) *Tephrosia vogelii, Tithonia diversifolia, Vernonia amygdalina,* and *Phytolacca dodecandra* plant extracts (PEs) are biologically active against *Tuta absoluta* with bioactivity ranging from medium to low in laboratory bioassays. The null hypothesis (iii) is therefore rejected.

iv) The entomopathogenic nematodes (*Steinernema* sp. RW14-M-C2a-3 and *Steinernema* sp. RW14-M-C2b-1), commercial formulations of EPFs (Metatech® WP: *M. anisopliae*, Strain FCM Ar 23B3 and Beauvitech® WP: *B. bassiana*, Strain J25) and azadirachtin are effective in reducing *T. absoluta* infestation to significantly lower levels under field conditions. However, field efficacy of the tested plant extracts is comparatively low. Consequently, the null hypothesis (iv) is rejected.

v) The entomopathogenic nematodes (*Steinernema* sp. RW14-M-C2a-3 and *Steinernema* sp. RW14-M-C2b-1), commercial formulations of EPFs (Metatech® WP: *M. anisopliae*, Strain FCM Ar 23B3 and Beauvitech® WP: *B. bassiana*, Strain J25) and azadirachtin significantly enhance tomato growth and yield, without compromising fruit quality. Field application of the studied plant extracts results in poor tomato growth and yield. Hence, the null hypothesis (v) is rejected.

8.3 Recommendations

Based on the results of this study, the following recommendations are formulated:

i) The entomopathogenic nematode (EPN) isolates from Rwanda, *Steinernema* sp. RW14-M-C2a-3 and *Steinernema* sp. RW14-M-C2b-1, should be considered for inclusion in IPM of *T. absoluta* in Rwanda.

ii) The commercial formulations of entomopathogenic fungi (EPFs), Metatech® WP (*M. anisopliae*, Strain FCM Ar 23B3) and Beauvitech® WP (*B. bassiana*, Strain J25), can be used against *T. absoluta*.

iii) *Tephrosia vogelii, T. diversifolia, V. amygdalina*, and *P. dodecandra* plant extracts (PEs) should be further evaluated to enhance their bioactivity against *T. absoluta*.

iv) *Steinernema* sp. RW14-M-C2a-3, *Steinernema* sp. RW14-M-C2b-, Metatech® WP (*M. anisopliae*, Strain FCM Ar 23B3), Beauvitech® WP (*B. bassiana*, Strain J25) and azadirachtin should be included in IPM of *T. absoluta* under field conditions in the study site.

v) Tomato growers can use *Steinernema* sp. RW14-M-C2a-3, *Steinernema* sp. RW14-M-C2b-, Metatech® WP (*M. anisopliae*, Strain FCM Ar 23B3), Beauvitech® WP (*B. bassiana*, Strain J25) and azadirachtin to enhance tomato growth and yield, without compromising fruit quality.

8.4 Areas for further studies

The following areas for further research are highlighted from the current study:

i) Greenhouse efficacy of entomopathogenic nematodes: *Steinernema* sp. RW14-M-C2a-3 and *Steinernema* sp. RW14-M-C2b-1, commercial formulations of EPFs: Metatech® WP (*M. anisopliae*, Strain FCM Ar 23B3) and Beauvitech® WP (*B. bassiana*, Strain J25) and azadirachtin against *T. absoluta*.

ii) Isolation of new EPNs from Rwanda and evaluation of their efficacy against *T. absoluta* to obtain the most virulent.

iii) Efficacy of different strains of commercial formulations of entomopathogenic fungi (other than strains evaluated in this study) against *T. absoluta*.

iv) Isolation of native strains of *B. bassiana* and *M. anisopliae* that are more adapted to natural conditions of Rwanda and evaluation of their efficacy against *T. absoluta*.

v) Efficacy of extracts from *T. vogelii, T. diversifolia, V. amygdalina*, and *P. dodecandra against T. absoluta* using different concentations and solvents.

vi) Development and optimisation of different formulations and application equipment to boost the efficacy of entomopathogens in management of *T. absoluta*.

vii) Compatibility and synergism of combined management options against *T. absoluta*.

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APPENDICES

Appendix A: *In-vivo* method for mass production of entomopathogenic nematodes

Appendix B: Key Data analysis output for objective one

```
proc means data=EPN_BIOASSAY_ONE mean std;
class Treatment;
var DayOne DaYTwo DayThree DayFour;
run;

data EPN_BIOASSAY_ONE; set EPN_BIOASSAY_ONE;
asinDayTwo=arsin(sqrt(DayTwo/100));
asinDayThree=arsin(sqrt(DayThree/100));
run;

proc glm data=EPN_BIOASSAY_ONE;
class Treatment;
model DayOne asinDayTwo asinDayThree DayFour=Treatment;
means Treatment/tukey;
run;
```

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The MEANS Procedure

Treatment	Obs	Variable	Mean	Std Dev
ALL	3	DayOne	6.6666667	5.7735027
		DayTwo	40.0000000	10.0000000
		DayThree	84.7333333	6.0451082
		DayFour	95.8333333	7.2168784
G-R3a-2	3	DayOne	90.6666667	5.7735027
		DayTwo	95.8333333	7.2168784
		DayThree	100.0000000	0
		DayFour	100.0000000	0
H06	3	DayOne	26.6666667	5.7735027
		DayTwo	85.8333333	5.2041650
		DayThree	100.0000000	0
		DayFour	100.0000000	0
M-C2a-3	3	DayOne	80.0000000	10.0000000
		DayTwo	96.6666667	5.7735027
		DayThree	100.0000000	0
		DayFour	100.0000000	0
M-C2b-1	3	DayOne	83.3333333	15.2752523
		DayTwo	92.5000000	6.6143783
		DayThree	100.0000000	0
		DayFour	100.0000000	0
N-C4a	3	DayOne	53.3333333	5.7735027
		DayTwo	96.6666667	5.7735027
		DayThree	100.0000000	0
		DayFour	100.0000000	0

The GLM Procedure Dependent Variable: DayOne

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	18777.77778	3755.55556	48.29	<.0001
Error	12	933.33333	77.77778		
Corrected Total	17	19711.11111			

R-Square	Coeff Var	Root MSE	DayOne Mean
0.952649	15.26395	8.819171	57.77778

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	5	18777.77778	3755.55556	48.29	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	5	18777.77778	3755.55556	48.29	<.0001

The GLM Procedure Dependent Variable: asinDayTwo

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	4566.062441	913.212488	9.95	0.0006
Error	12	1101.680799	91.806733		
Corrected Total	17	5667.743240			

R-Square	Coeff Var	Root MSE	asinDayTwo Mean
0.805623	13.21477	9.581583	72.50663

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	5	4566.062441	913.212488	9.95	0.0006

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	5	4566.062441	913.212488	9.95	0.0006

The GLM Procedure Dependent Variable: asinDayThree

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	1295.086495	259.017299	71.06	<.0001
Error	12	43.737581	3.644798		
Corrected Total	17	1338.824076			

R-Square	Coeff Var	Root MSE	asinDayThree Mean
0.967331	2.214434	1.909136	86.21323

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	5	1295.086495	259.017299	71.06	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	5	1295.086495	259.017299	71.06	<.0001

The GLM Procedure Dependent Variable: DayFour

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	43.4027778	8.6805556	1.00	0.4582
Error	12	104.1666667	8.6805556		
Corrected Total	17	147.5694444			

R-Square	Coeff Var	Root MSE	DayFour Mean
0.294118	2.966882	2.946278	99.30556

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	5	43.40277778	8.68055556	1.00	0.4582

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	5	43.40277778	8.68055556	1.00	0.4582

Tukey's Studentized Range (HSD) Test for DayOne

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	77.77778
Critical Value of Studentized Range	4.75015
Minimum Significant Difference	24.187

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Treatment
A	90.667	3	G-R3a-2
A	83.333	3	M-C2b-1
A	80.000	3	M-C2a-3
B	53.333	3	N-C4a
C	26.667	3	H06
C	6.667	3	ALL

Tukey's Studentized Range (HSD) Test for asinDayTwo

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	91.80673
Critical Value of Studentized Range	4.75015
Minimum Significant Difference	26.278

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Treatment
A	83.861	3	M-C2a-3
A	83.861	3	N-C4a
A	83.105	3	G-R3a-2
A	76.959	3	M-C2b-1
A	68.103	3	HO6
B	39.150	3	ALL

Tukey's Studentized Range (HSD) Test for asinDayThree

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	3.644798
Critical Value of Studentized Range	4.75015
Minimum Significant Difference	5.2358

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Treatment
A	90.007	3	M-C2b-1
A	90.007	3	G-R3a-2
A	90.007	3	HO6
A	90.007	3	M-C2a-3
A	90.007	3	N-C4a
B	67.246	3	ALL

Tukey's Studentized Range (HSD) Test for DayFour

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	8.680556
Critical Value of Studentized Range	4.75015
Minimum Significant Difference	8.0802

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Treatment
A	100.000	3	M-C2b-1
A	100.000	3	G-R3a-2
A	100.000	3	HO6
A	100.000	3	M-C2a-3
A	100.000	3	N-C4a
A	95.833	3	ALL

```
proc GLM data=ALL_BIOASSAYS_COMBINED;
class EPN H;
model asinBioassayOne asinBioassayTwo asinBioassayThree=EPN H EPN*H;
run;
```

Class Level Information

Class	Levels	Values
EPN	6	ALL G-R3a-2 H06 M-C2a-3 M-C2b-1 N-C4a
H	4	24h 48h 72h 96h
Number of observations	72	

The GLM Procedure Dependent Variable: asinBioassayOne

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	23	9.88396620	0.42973766	25.43	<.0001
Error	48	0.81117907	0.01689956		
Corrected Total	71	10.69514527			

R-Square	Coeff Var	Root MSE	asinBioassayOne Mean
0.924154	9.967604	0.129998	1.304208

Source	DF	Type I SS	Mean Square	F Value	Pr > F
EPN	5	3.17583222	0.63516644	37.58	<.0001
H	3	4.84119506	1.61373169	95.49	<.0001
EPN*H	15	1.86693892	0.12446259	7.36	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
EPN	5	3.17583222	0.63516644	37.58	<.0001
H	3	4.84119506	1.61373169	95.49	<.0001
EPN*H	15	1.86693892	0.12446259	7.36	<.0001

The GLM Procedure Dependent Variable: asinBioassayTwo

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	23	12.57444458	0.54671498	32.61	<.0001
Error	48	0.80465012	0.01676354		
Corrected Total	71	13.37909470			

R-Square	Coeff Var	Root MSE	asinBioassayTwo Mean
0.939858	9.907429	0.129474	1.306839

Source	DF	Type I SS	Mean Square	F Value	Pr > F
EPN	5	4.56192955	0.91238591	54.43	<.0001
H	3	4.77974246	1.59324749	95.04	<.0001
EPN*H	15	3.23277257	0.21551817	12.86	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
EPN	5	4.56192955	0.91238591	54.43	<.0001
H	3	4.77974246	1.59324749	95.04	<.0001
EPN*H	15	3.23277257	0.21551817	12.86	<.0001

The GLM Procedure Dependent Variable: asinBioassayThree

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	23	13.19456731	0.57367684	50.98	<.0001
Error	48	0.54011677	0.01125243		
Corrected Total	71	13.73468408			

R-Square	Coeff Var	Root MSE	asinBioassayThree Mean
0.960675	8.217692	0.106077	1.290843

Source	DF	Type I SS	Mean Square	F Value	Pr > F
EPN	5	4.75088071	0.95017614	84.44	<.0001
H	3	5.19024376	1.73008125	153.75	<.0001
EPN*H	15	3.25344284	0.21689619	19.28	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
EPN	5	4.75088071	0.95017614	84.44	<.0001
H	3	5.19024376	1.73008125	153.75	<.0001
EPN*H	15	3.25344284	0.21689619	19.28	<.0001

Appendix C: Key data analysis output for objective two

```
proc means data=EPF_BIOASSAY_TWO mean std;
class Treatment;
var DayFour DayFive DaySix;
run;
data EPF_BIOASSAY_TWO; set EPF_BIOASSAY_TWO;
logDayFive=log(DayFive+1);
logDaySix=log(DaySix+1);
run;
proc glm data=EPF_BIOASSAY_TWO;
class Treatment;
model DayFour logDayFive logDaySix=Treatment;
means treatment/tukey;
run;
```

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The MEANS Procedure

Treatment	Obs	Variable	Mean	Std Dev
Beauvite	4	DayFour	31.5000000	1.7320508
		DayFive	47.5000000	7.0000000
		DaySix	58.0000000	6.2716292
Botaniga	4	DayFour	23.7500000	6.2383224
		DayFive	36.7500000	6.3966137
		DaySix	45.5000000	5.7445626
Imidaclo	4	DayFour	10.0000000	11.5470054
		DayFive	28.2500000	14.2916059
		DaySix	27.5000000	7.1414284
Metatech	4	DayFour	55.0000000	7.5718778
		DayFive	73.7500000	6.2383224
		DaySix	79.5000000	10.3440804

The GLM Procedure Dependent Variable: DayFour

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	4265.187500	1421.729167	24.45	<.0001
Error	12	697.750000	58.145833		
Corrected Total	15	4962.937500			

R-Square	Coeff Var	Root MSE	DayFour Mean
0.859408	25.36496	7.625342	30.06250

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	3	4265.187500	1421.729167	24.45	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	3	4265.187500	1421.729167	24.45	<.0001

The GLM Procedure Dependent Variable: logDayFive

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	2.33844322	0.77948107	7.81	0.0037
Error	12	1.19795794	0.09982983		
Corrected Total	15	3.53640116			

R-Square	Coeff Var	Root MSE	logDayFive Mean
0.661249	8.387911	0.315959	3.766833

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	3	2.33844322	0.77948107	7.81	0.0037

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	3	2.33844322	0.77948107	7.81	0.0037

The GLM Procedure Dependent Variable: logDaySix

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	2.37119339	0.79039780	32.07	<.0001
Error	12	0.29571902	0.02464325		
Corrected Total	15	2.66691241			

R-Square	Coeff Var	Root MSE	logDaySix Mean
0.889116	4.020707	0.156982	3.904330

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	3	2.37119339	0.79039780	32.07	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	3	2.37119339	0.79039780	32.07	<.0001

Tukey's Studentized Range (HSD) Test for DayFour

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	58.14583
Critical Value of Studentized Range	4.19852
Minimum Significant Difference	16.008

Means with the same letter are not significantly different.

Tukey Grouping		Mean	N	Treatment
	A	55.000	4	Metatech
	B	31.500	4	Beauvite
C	B	23.750	4	Botaniga
C		10.000	4	Imidaclo

Tukey's Studentized Range (HSD) Test for logDayFive

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	0.09983
Critical Value of Studentized Range	4.19852
Minimum Significant Difference	0.6633

Means with the same letter are not significantly different.

Tukey Grouping		Mean	N	Treatment
	A	4.3115	4	Metatech
B	A	3.8738	4	Beauvite
B		3.6201	4	Botaniga
B		3.2619	4	Imidaclo

Tukey's Studentized Range (HSD) Test for logDaySix

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	0.024643
Critical Value of Studentized Range	4.19852
Minimum Significant Difference	0.3295

Means with the same letter are not significantly different.

Tukey Grouping		Mean	N	Treatment
	A	4.3819	4	Metatech
B	A	4.0732	4	Beauvite
B		3.8335	4	Botaniga
	C	3.3288	4	Imidaclo

```
proc means data=LC50_VALUES mean std;
class Treatment;
var BioassayOne BioassayTwo BioassayThree Pooled;
run;

data LC50_VALUES; set LC50_VALUES;
rsqrBioassayOne=1/(sqrt(BioassayOne));
rsqrBioassayTwo=1/(sqrt(BioassayTwo));
rsqrBioassayThree=1/(sqrt(BioassayThree));
rsqrPooled=1/(sqrt(Pooled));
run;

proc glm data=LC50_VALUES;
class Treatment;
model rsqrBioassayOne rsqrBioassayTwo rsqrBioassayThree rsqrPooled=Treatment;
means treatment/tukey;
run
```

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The MEANS Procedure

Treatment	Obs	Variable	Mean	Std Dev
Beauvite	4	BioassayOne	5.1875000	0.1562850
		BioassayTwo	5.1850000	0.4036913
		BioassayThree	5.3375000	0.3254100
		Pooled	5.2350000	0.2568398
Botaniga	4	BioassayOne	6.2475000	0.3662763
		BioassayTwo	6.7525000	1.0363196
		BioassayThree	6.6875000	1.7630159
		Pooled	6.5625000	0.5122743
Imidaclo	4	BioassayOne	14.0900000	7.7630192
		BioassayTwo	16.1075000	6.5278653
		BioassayThree	14.4275000	9.6156275
		Pooled	14.8750000	2.8009582
Metatech	4	BioassayOne	4.2275000	0.2964653
		BioassayTwo	3.4650000	0.4795484
		BioassayThree	4.0175000	0.6021835
		Pooled	3.9050000	0.1717556

The GLM Procedure Dependent Variable: rsqrBioassayOne

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.08072886	0.02690962	13.10	0.0004
Error	12	0.02464606	0.00205384		
Corrected Total	15	0.10537492			

R-Square	Coeff Var	Root MSE	rsqrBioassayOne Mean
0.766111	11.18323	0.045319	0.405243

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	3	0.08072886	0.02690962	13.10	0.0004

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	3	0.08072886	0.02690962	13.10	0.0004

The GLM Procedure Dependent Variable: rsqrBioassayTwo

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.15482594	0.05160865	25.19	<.0001
Error	12	0.02459013	0.00204918		
Corrected Total	15	0.17941607			

R-Square	Coeff Var	Root MSE	rsqrBioassayTwo Mean
0.862944	11.07906	0.045268	0.408589

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	3	0.15482594	0.05160865	25.19	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	3	0.15482594	0.05160865	25.19	<.0001

The GLM Procedure Dependent Variable: rsqrBioassayThree

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.08128611	0.02709537	6.93	0.0058
Error	12	0.04690897	0.00390908		
Corrected Total	15	0.12819508			

R-Square	Coeff Var	Root MSE	rsqrBioassayThree Mean
0.634081	15.30577	0.062523	0.408491

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	3	0.08128611	0.02709537	6.93	0.0058

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	3	0.08128611	0.02709537	6.93	0.0058

The GLM Procedure Dependent Variable: rsqrPooled

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.12717572	0.04239191	144.06	<.0001
Error	12	0.00353112	0.00029426		
Corrected Total	15	0.13070685			

R-Square	Coeff Var	Root MSE	rsqrPooled Mean
0.972984	4.297061	0.017154	0.399203

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	3	0.12717572	0.04239191	144.06	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	3	0.12717572	0.04239191	144.06	<.0001

Tukey's Studentized Range (HSD) Test for rsqrBioassayOne

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	0.002054
Critical Value of Studentized Range	4.19852
Minimum Significant Difference	0.0951

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Treatment
A	0.48707	4	Metatech
A	0.43917	4	Beauvite
A	0.40046	4	Botaniga
B	0.29428	4	Imidaclo

Tukey's Studentized Range (HSD) Test for rsqrBioassayTwo

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	0.002049
Critical Value of Studentized Range	4.19852
Minimum Significant Difference	0.095

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Treatment
A	0.53998	4	Metatech
B	0.43993	4	Beauvite
B	0.38729	4	Botaniga
C	0.26715	4	Imidaclo

Tukey's Studentized Range (HSD) Test for rsqrBioassayThree

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	0.003909
Critical Value of Studentized Range	4.19852
Minimum Significant Difference	0.1313

Means with the same letter are not significantly different.

Tukey Grouping		Mean	N	Treatment
	A	0.50223	4	Metatech
B	A	0.43330	4	Beauvite
B	A	0.39333	4	Botaniga
B		0.30510	4	Imidaclo

Tukey's Studentized Range (HSD) Test for rsqrPooled

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	0.000294
Critical Value of Studentized Range	4.19852
Minimum Significant Difference	0.036

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Treatment
A	0.50632	4	Metatech
B	0.43737	4	Beauvite
C	0.39102	4	Botaniga
D	0.26210	4	Imidaclo

Appendix D: Key data analysis output for objective three

```
proc means
data=EXTRACT_BIOASSAY_Three mean std;
class Treatment;
var DayOne DayTwo DayThree DayFour DayFive;
run;

data EXTRACT_BIOASSAY_Three; set EXTRACT_BIOASSAY_Three;
asinDayOne=arsin(sqrt(DayOne/100));
asinDayTwo=arsin(sqrt(DayTwo/100));
asinDayThree=arsin(sqrt(DayThree/100));
asinDayFour=arsin(sqrt(DayFour/100));
asinDayFive=arsin(sqrt(DayFive/100));
run;

proc glm data=EXTRACT_BIOASSAY_Three;
class Treatment;
model asinDayOne asinDayTwo asinDayThree asinDayFour asinDayFive=Treatment;
means treatment/tukey;
run;
```

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Treatment	Obs	Variable	Mean	Std Dev
Azadirac	4	DayOne	37.5000000	9.5742711
		DayTwo	75.0000000	5.7735027
		DayThree	87.2250000	4.8444986
		DayFour	94.7250000	6.1075773
		DayFive	97.5000000	5.0000000
P.dodeca	4	DayOne	0	0
		DayTwo	7.5000000	5.0000000
		DayThree	18.0500000	5.4659552
		DayFour	18.3250000	4.9270512
		DayFive	24.8500000	3.6783148
T.divers	4	DayOne	0	0
		DayTwo	0	0
		DayThree	2.5000000	5.0000000
		DayFour	5.0000000	5.7735027
		DayFive	5.2750000	6.1075773
T.vogeli	4	DayOne	10.0000000	0
		DayTwo	17.5000000	5.0000000
		DayThree	20.2750000	7.7224241
		DayFour	31.3750000	7.3974658
		DayFive	33.2500000	7.8682908
V.amygda	4	DayOne	0	0
		DayTwo	0	0
		DayThree	2.5000000	5.0000000
		DayFour	2.5000000	5.0000000
		DayFive	2.5000000	5.0000000

The GLM Procedure Dependent Variable: asinDayOne

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	1.37561654	0.34390414	176.52	<.0001
Error	15	0.02922317	0.00194821		
Corrected Total	19	1.40483971			

R-Square	Coeff Var	Root MSE	asinDayOne Mean
0.979198	22.54037	0.044139	0.195820

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	4	1.37561654	0.34390414	176.52	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	4	1.37561654	0.34390414	176.52	<.0001

The GLM Procedure Dependent Variable: asinDayTwo

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	3.00616673	0.75154168	106.15	<.0001
Error	15	0.10619782	0.00707985		
Corrected Total	19	3.11236455			

R-Square	Coeff Var	Root MSE	asinDayTwo Mean
0.965879	24.47922	0.084142	0.343728

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	4	3.00616673	0.75154168	106.15	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	4	3.00616673	0.75154168	106.15	<.0001

The GLM Procedure Dependent Variable: asinDayThree

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	3.39874055	0.84968514	59.08	<.0001
Error	15	0.21572706	0.01438180		
Corrected Total	19	3.61446762			

R-Square	Coeff Var	Root MSE	asinDayThree Mean
0.940316	26.45541	0.119924	0.453307

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	4	3.39874055	0.84968514	59.08	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	4	3.39874055	0.84968514	59.08	<.0001

The GLM Procedure Dependent Variable: asinDayFour

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	4.46686248	1.11671562	51.70	<.0001
Error	15	0.32400151	0.02160010		
Corrected Total	19	4.79086400			

R-Square	Coeff Var	Root MSE	asinDayFour Mean
0.932371	27.43329	0.146970	0.535735

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	4	4.46686248	1.11671562	51.70	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	4	4.46686248	1.11671562	51.70	<.0001

The GLM Procedure Dependent Variable: asinDayFive

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	5.01831700	1.25457925	64.37	<.0001
Error	15	0.29234925	0.01948995		
Corrected Total	19	5.31066625			

R-Square	Coeff Var	Root MSE	asinDayFive Mean
0.944951	24.32467	0.139606	0.573929

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	4	5.01831700	1.25457925	64.37	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	4	5.01831700	1.25457925	64.37	<.0001

Tukey's Studentized Range (HSD) Test for asinDayOne

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	15
Error Mean Square	0.001948
Critical Value of Studentized Range	4.36699
Minimum Significant Difference	0.0964

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Treatment
A	0.65735	4	Azadirac
B	0.32175	4	T.vogeli
C	0.00000	4	T.divers
C	0.00000	4	P. dodeca
C	0.00000	4	V. amygda

Tukey's Studentized Range (HSD) Test for asinDayTwo

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	15
Error Mean Square	0.00708
Critical Value of Studentized Range	4.36699
Minimum Significant Difference	0.1837

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Treatment
A	1.04915	4	Azadirac
B	0.42817	4	T.vogeli
C	0.24131	4	P.dodeca
D	0.00000	4	T.divers
D	0.00000	4	V.amygda

Tukey's Studentized Range (HSD) Test for asinDayThree

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	15
Error Mean Square	0.014382
Critical Value of Studentized Range	4.36699
Minimum Significant Difference	0.2619

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Treatment
A	1.20909	4	Azadirac
B	0.46165	4	T.vogeli
B	0.43492	4	P.dodeca
C	0.08044	4	T.divers
C	0.08044	4	V.amygda

Tukey's Studentized Range (HSD) Test for asinDayFour

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	15
Error Mean Square	0.0216
Critical Value of Studentized Range	4.36699
Minimum Significant Difference	0.3209

Means with the same letter are not significantly different.

Tukey Grouping		Mean	N	Treatment
A		1.4054	4	Azadirac
	B	0.5925	4	T.vogeli
C	B	0.4394	4	P.dodeca
C	D	0.1609	4	T.divers
	D	0.0804	4	V.amygda

Tukey's Studentized Range (HSD) Test for asinDayFive

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	15
Error Mean Square	0.01949
Critical Value of Studentized Range	4.36699
Minimum Significant Difference	0.3048

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Treatment
A	1.49036	4	Azadirac
B	0.61238	4	T.vogeli
B	0.52112	4	P.dodeca
C	0.16535	4	T.divers
C	0.08044	4	V.amygda

Appendix E: Key data analysis output for objective four

```
proc glm data=Leafdamage_Trial_Two;
class Block Treatment;
model logTWO logTHREE logFOUR logFIVE logSIX SEVENWAT EIGHTWAT NINEWAT=Block
Treatment;
means Treatment/TUKEY;
run;
```

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Treatment	Obs	Variable	Mean	Std Dev
T1	3	TWOWAT	6.9333333	3.0924640
		THREEWAT	11.3666667	4.1198705
		FOURWAT	18.9333333	6.6643329
		FIVEWAT	45.2000000	6.5092242
		SIXWAT	63.9333333	3.0072135
		SEVENWAT	81.8666667	0.2886751
		EIGHTWAT	98.3333333	2.6312228
		NINEWAT	100.0000000	0
T2	3	TWOWAT	6.9666667	2.6312228
		THREEWAT	14.7333333	3.8682468
		FOURWAT	21.8000000	3.4073450
		FIVEWAT	42.0000000	6.8417834
		SIXWAT	66.2333333	2.3158872
		SEVENWAT	83.1666667	1.2741010
		EIGHTWAT	98.4000000	1.6000000
		NINEWAT	100.0000000	0
T3	3	TWOWAT	6.9666667	2.6312228
		THREEWAT	18.0333333	3.5232561
		FOURWAT	20.3000000	3.9585351
		FIVEWAT	47.9333333	2.1548395
		SIXWAT	70.5000000	1.5716234
		SEVENWAT	83.2666667	1.1846237
		EIGHTWAT	100.0000000	0
		NINEWAT	100.0000000	0
T4	3	TWOWAT	5.5000000	0.4582576
		THREEWAT	15.1333333	4.3247351
		FOURWAT	23.8666667	5.6092186
		FIVEWAT	47.3333333	4.0918618
		SIXWAT	72.9000000	5.3225934
		SEVENWAT	83.9000000	0.2000000
		EIGHTWAT	99.4666667	0.9237604
		NINEWAT	100.0000000	0
T5	3	TWOWAT	6.8666667	3.1564748
		THREEWAT	21.0333333	2.2188586
		FOURWAT	44.6333333	6.5041013
		FIVEWAT	55.9666667	7.4741778
		SIXWAT	71.4666667	4.3821608
		SEVENWAT	88.3000000	3.8223030
		EIGHTWAT	100.0000000	0
		NINEWAT	100.0000000	0
T6	3	TWOWAT	7.0333333	3.0022214
		THREEWAT	27.1333333	3.0989245
		FOURWAT	53.9000000	1.6522712
		FIVEWAT	80.1333333	2.0792627
		SIXWAT	85.2333333	4.1137979
		SEVENWAT	95.1666667	6.2564633
		EIGHTWAT	100.0000000	0
		NINEWAT	100.0000000	0
T7	3	TWOWAT	6.8666667	2.7135463

		THREEWAT	17.0333333	7.0116570
		FOURWAT	23.4000000	8.4640416
		FIVEWAT	46.3333333	7.3711148
		SIXWAT	67.5000000	2.2605309
		SEVENWAT	83.1666667	2.4501701
		EIGHTWAT	96.8333333	2.9737743
		NINEWAT	100.0000000	0
T8	3	TWOWAT	7.2333333	2.8290163
		THREEWAT	28.1666667	8.2706308
		FOURWAT	53.2333333	3.2005208
		FIVEWAT	78.4666667	3.7447741
		SIXWAT	83.3333333	8.6858122
		SEVENWAT	91.8000000	2.6851443
		EIGHTWAT	100.0000000	0
		NINEWAT	100.0000000	0
T9	3	TWOWAT	6.9000000	3.1192948
		THREEWAT	29.3333333	7.5035547
		FOURWAT	60.9000000	5.9025418
		FIVEWAT	84.9000000	2.6057628
		SIXWAT	86.8000000	3.7722672
		SEVENWAT	96.6000000	3.2186954
		EIGHTWAT	100.0000000	0
		NINEWAT	100.0000000	0

The GLM Procedure Dependent Variable: logTwo

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	0.10381583	0.01038158	0.09	0.9997
Error	16	1.83321321	0.11457583		
Corrected Total	26	1.93702904			

R-Square	Coeff Var	Root MSE	logTwo Mean
0.053595	16.78331	0.338491	2.016829

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Block	2	0.02401067	0.01200533	0.10	0.9011
Treatment	8	0.07980517	0.00997565	0.09	0.9992

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Block	2	0.02401067	0.01200533	0.10	0.9011
Treatment	8	0.07980517	0.00997565	0.09	0.9992

The GLM Procedure Dependent Variable: logThree

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	2.78454962	0.27845496	3.42	0.0142
Error	16	1.30440024	0.08152502		
Corrected Total	26	4.08894986			

R-Square	Coeff Var	Root MSE	logThree Mean
0.680994	9.731709	0.285526	2.933974

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Block	2	0.03703781	0.01851891	0.23	0.7993
Treatment	8	2.74751180	0.34343898	4.21	0.0070

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Block	2	0.03703781	0.01851891	0.23	0.7993
Treatment	8	2.74751180	0.34343898	4.21	0.0070

The GLM Procedure Dependent Variable: logFour

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	5.95357156	0.59535716	11.58	<.0001
Error	16	0.82282253	0.05142641		
Corrected Total	26	6.77639409			

R-Square	Coeff Var	Root MSE	logFour Mean
0.878575	6.565532	0.226774	3.454007

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Block	2	0.04977661	0.02488830	0.48	0.6251
Treatment	8	5.90379495	0.73797437	14.35	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Block	2	0.04977661	0.02488830	0.48	0.6251
Treatment	8	5.90379495	0.73797437	14.35	<.0001

The GLM Procedure Dependent Variable: logFive

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	1.93216223	0.19321622	14.60	<.0001
Error	16	0.21179421	0.01323714		
Corrected Total	26	2.14395644			

R-Square	Coeff Var	Root MSE	logFive Mean
0.901213	2.853404	0.115053	4.032123

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Block	2	0.00068928	0.00034464	0.03	0.9743
Treatment	8	1.93147295	0.24143412	18.24	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Block	2	0.00068928	0.00034464	0.03	0.9743
Treatment	8	1.93147295	0.24143412	18.24	<.0001

The GLM Procedure Dependent Variable: logSix

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	0.31479011	0.03147901	8.76	<.0001
Error	16	0.05747311	0.00359207		
Corrected Total	26	0.37226322			

R-Square	Coeff Var	Root MSE	logSix Mean
0.845612	1.393841	0.059934	4.299908

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Block	2	0.00115020	0.00057510	0.16	0.8534
Treatment	8	0.31363991	0.03920499	10.91	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Block	2	0.00115020	0.00057510	0.16	0.8534
Treatment	8	0.31363991	0.03920499	10.91	<.0001

The GLM Procedure Dependent Variable: SEVENWAT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	829.9703704	82.9970370	11.69	<.0001
Error	16	113.6259259	7.1016204		
Corrected Total	26	943.5962963			

R-Square	Coeff Var	Root MSE	SEVENWAT Mean
0.879582	3.046616	2.664887	87.47037

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Block	2	47.3274074	23.6637037	3.33	0.0617
Treatment	8	782.6429630	97.8303704	13.78	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Block	2	47.3274074	23.6637037	3.33	0.0617
Treatment	8	782.6429630	97.8303704	13.78	<.0001

The GLM Procedure Dependent Variable: EIGHTWAT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	39.15925926	3.91592593	2.09	0.0910
Error	16	29.97259259	1.87328704		
Corrected Total	26	69.13185185			

R-Square	Coeff Var	Root MSE	EIGHTWAT Mean
0.566443	1.379358	1.368681	99.22593

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Block	2	8.38740741	4.19370370	2.24	0.1389
Treatment	8	30.77185185	3.84648148	2.05	0.1050

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Block	2	8.38740741	4.19370370	2.24	0.1389
Treatment	8	30.77185185	3.84648148	2.05	0.1050

The GLM Procedure Dependent Variable: NINEWAT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	0	0	.	.
Error	16	0	0		
Corrected Total	26	0			

R-Square	Coeff Var	Root MSE	NINEWAT Mean
0.000000	0	0	100.0000

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Block	2	0	0	.	.
Treatment	8	0	0	.	.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Block	2	0	0	.	.
Treatment	8	0	0	.	.

Tukey's Studentized Range (HSD) Test for logTwo

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0.114576
Critical Value of Studentized Range	5.03101
Minimum Significant Difference	0.9832

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Treatment
A	2.0722	3	T8
A	2.0418	3	T3
A	2.0418	3	T2
A	2.0411	3	T6
A	2.0263	3	T7
A	2.0249	3	T1
A	2.0196	3	T9
A	2.0136	3	T5
A	1.8701	3	T4

Tukey's Studentized Range (HSD) Test for logThree

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0.081525
Critical Value of Studentized Range	5.03101

Minimum Significant Difference 0.8294

Means with the same letter are not significantly different.

Tukey Grouping		Mean	N	Treatment
	A	3.3560	3	T9
	A	3.3054	3	T8
	A	3.2965	3	T6
B	A	3.0425	3	T5
B	A	2.8789	3	T3
B	A	2.7824	3	T7
B	A	2.6895	3	T4
B	A	2.6688	3	T2
B		2.3858	3	T1

Tukey's Studentized Range (HSD) Test for logFour

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0.051426
Critical Value of Studentized Range	5.03101
Minimum Significant Difference	0.6587

Means with the same letter are not significantly different.

Tukey Grouping		Mean	N	Treatment
	A	4.1061	3	T9
	A	3.9868	3	T6
	A	3.9735	3	T8
B	A	3.7910	3	T5
B	C	3.1552	3	T4
	C	3.1085	3	T7
	C	3.0732	3	T2
	C	2.9975	3	T3
	C	2.8942	3	T1

Tukey's Studentized Range (HSD) Test for logFive

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0.013237
Critical Value of Studentized Range	5.03101
Minimum Significant Difference	0.3342

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Treatment
A	4.44116	3	T9
A	4.38347	3	T6
A	4.36192	3	T8
B	4.01892	3	T5
B	3.86913	3	T3
B	3.85477	3	T4
B	3.82688	3	T7
B	3.80382	3	T1
B	3.72903	3	T2

Tukey's Studentized Range (HSD) Test for logSix

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0.003592
Critical Value of Studentized Range	5.03101
Minimum Significant Difference	0.1741

Means with the same letter are not significantly different.

Tukey Grouping			Mean	N	Treatment
	A		4.46298	3	T9
B	A		4.44462	3	T6
B	A	C	4.41913	3	T8
B	D	C	4.28735	3	T4
	D	C	4.26800	3	T5
	D	C	4.25545	3	T3
	D		4.21176	3	T7
	D		4.19278	3	T2
	D		4.15709	3	T1

Tukey's Studentized Range (HSD) Test for SEVENWAT

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	7.10162
Critical Value of Studentized Range	5.03101
Minimum Significant Difference	7.7406

Means with the same letter are not significantly different.

Tukey Grouping			Mean	N	Treatment
	A		96.600	3	T9
B	A		95.167	3	T6
B	A		91.800	3	T8
B	C		88.300	3	T5
	C		83.900	3	T4
	C		83.267	3	T3
	C		83.167	3	T7
	C		83.167	3	T2
	C		81.867	3	T1

Tukey's Studentized Range (HSD) Test for EIGHTWAT

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	1.873287
Critical Value of Studentized Range	5.03101
Minimum Significant Difference	3.9755

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Treatment
A	100.000	3	T9
A	100.000	3	T6
A	100.000	3	T3
A	100.000	3	T8
A	100.000	3	T5
A	99.467	3	T4
A	98.400	3	T2
A	98.333	3	T1
A	96.833	3	T7

Tukey's Studentized Range (HSD) Test for NINEWAT

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0
Critical Value of Studentized Range	5.03101
Minimum Significant Difference	0

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Treatment
A	100.0	3	T1
A	100.0	3	T2
A	100.0	3	T3
A	100.0	3	T4
A	100.0	3	T5
A	100.0	3	T6
A	100.0	3	T7
A	100.0	3	T8
A	100.0	3	T9

```
proc glm data=Leafletdamage_Trial_One;
class block treat;
model sqrTwo logThree logFour logFive logSix logSeven logEight logNine
logTen=block treat;
means treat/TUKEY;
run;
```

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The MEANS Procedure

Treat	N Obs	Variable	Mean	Std Dev
T1	3	TWOWAT	2.9333333	2.6102363
		THREEWAT	4.2333333	0.4163332
		FOURWAT	8.5000000	0.6082763
		FIVEWAT	13.3666667	0.2081666
		SIXWAT	18.3666667	1.3503086
		SEVENWAT	26.1000000	2.5534291
		EIGHTWAT	37.6333333	2.7646579
		NINEWAT	48.8000000	2.2000000
		TENWAT	59.6666667	4.8211340
T2	3	TWOWAT	2.9666667	2.5929391
		THREEWAT	4.7000000	0.4582576
		FOURWAT	8.9333333	0.6027714
		FIVEWAT	14.3000000	1.0000000
		SIXWAT	20.0666667	2.2030282
		SEVENWAT	27.9000000	3.7749172
		EIGHTWAT	38.7333333	1.3650397
		NINEWAT	51.4666667	1.4047538
		TENWAT	64.0666667	2.5794056
T3	3	TWOWAT	2.8666667	2.5794056
		THREEWAT	5.4666667	1.2055428
		FOURWAT	9.4000000	0.6557439
		FIVEWAT	13.6666667	0.5507571
		SIXWAT	19.3000000	1.2000000
		SEVENWAT	26.9000000	3.0347982
		EIGHTWAT	38.4666667	2.1501938
		NINEWAT	54.9000000	0.8185353
		TENWAT	63.5000000	0.6557439
T4	3	TWOWAT	2.2000000	3.8105118

		THREEWAT	5.7666667	0.8020806
		FOURWAT	9.2666667	0.5686241
		FIVEWAT	15.0333333	1.0969655
		SIXWAT	21.0666667	0.8144528
		SEVENWAT	26.4333333	0.3511885
		EIGHTWAT	39.4666667	1.7214335
		NINEWAT	54.5000000	3.5791060
		TENWAT	66.0000000	4.3714986
T5	3	TWOWAT	2.9666667	1.4843629
		THREEWAT	5.8333333	0.8020806
		FOURWAT	11.2333333	0.7505553
		FIVEWAT	18.0000000	0.7937254
		SIXWAT	26.9666667	1.1060440
		SEVENWAT	35.7000000	2.4020824
		EIGHTWAT	49.2666667	3.1469562

The GLM Procedure Dependent Variable: sqrTwo

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	0.48384842	0.04838484	0.06	1.0000
Error	16	12.84886646	0.80305415		
Corrected Total	26	13.33271488			

R-Square	Coeff Var	Root MSE	sqrTwo Mean
0.036290	54.40004	0.896133	1.647302

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Block	2	0.07505732	0.03752866	0.05	0.9545
Treat	8	0.40879109	0.05109889	0.06	0.9997

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Block	2	0.07505732	0.03752866	0.05	0.9545
Treat	8	0.40879109	0.05109889	0.06	0.9997

The GLM Procedure Dependent Variable: logThree

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	1.40985501	0.14098550	3.79	0.0089
Error	16	0.59594626	0.03724664		
Corrected Total	26	2.00580126			

R-Square	Coeff Var	Root MSE	logThree Mean
0.702889	11.04702	0.192994	1.747022

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Block	2	0.01083762	0.00541881	0.15	0.8657
Treat	8	1.39901739	0.17487717	4.70	0.0042

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Block	2	0.01083762	0.00541881	0.15	0.8657
Treat	8	1.39901739	0.17487717	4.70	0.0042

The GLM Procedure Dependent Variable: logFour

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	1.69682308	0.16968231	52.86	<.0001
Error	16	0.05135602	0.00320975		
Corrected Total	26	1.74817911			

R-Square	Coeff Var	Root MSE	logFour Mean
0.970623	2.385351	0.056655	2.375108

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Block	2	0.01859195	0.00929597	2.90	0.0844
Treat	8	1.67823113	0.20977889	65.36	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Block	2	0.01859195	0.00929597	2.90	0.0844
Treat	8	1.67823113	0.20977889	65.36	<.0001

The GLM Procedure Dependent Variable: logFive

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	1.45649247	0.14564925	60.15	<.0001
Error	16	0.03874524	0.00242158		
Corrected Total	26	1.49523772			

R-Square	Coeff Var	Root MSE	logFive Mean
0.974088	1.745212	0.049210	2.819688

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Block	2	0.00313756	0.00156878	0.65	0.5364
Treat	8	1.45335491	0.18166936	75.02	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Block	2	0.00313756	0.00156878	0.65	0.5364
Treat	8	1.45335491	0.18166936	75.02	<.0001

The GLM Procedure Dependent Variable: logSix

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	1.16897855	0.11689785	23.13	<.0001
Error	16	0.08087560	0.00505473		
Corrected Total	26	1.24985415			

R-Square	Coeff Var	Root MSE	logSix Mean
0.935292	2.250643	0.071097	3.158945

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Block	2	0.01925892	0.00962946	1.91	0.1811
Treat	8	1.14971963	0.14371495	28.43	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Block	2	0.01925892	0.00962946	1.91	0.1811
Treat	8	1.14971963	0.14371495	28.43	<.0001

The GLM Procedure Dependent Variable: logSeven

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	1.19001748	0.11900175	12.93	<.0001
Error	16	0.14726381	0.00920399		
Corrected Total	26	1.33728129			

R-Square	Coeff Var	Root MSE	logSeven Mean
0.889878	2.767471	0.095937	3.466610

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Block	2	0.01290541	0.00645271	0.70	0.5107
Treat	8	1.17711207	0.14713901	15.99	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Block	2	0.01290541	0.00645271	0.70	0.5107
Treat	8	1.17711207	0.14713901	15.99	<.0001

The GLM Procedure Dependent Variable: logEight

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	1.39956883	0.13995688	37.07	<.0001
Error	16	0.06041285	0.00377580		
Corrected Total	26	1.45998169			

R-Square	Coeff Var	Root MSE	logEight Mean
0.958621	1.600055	0.061448	3.840341

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Block	2	0.00151007	0.00075504	0.20	0.8208
Treat	8	1.39805876	0.17475735	46.28	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Block	2	0.00151007	0.00075504	0.20	0.8208
Treat	8	1.39805876	0.17475735	46.28	<.0001

The GLM Procedure Dependent Variable: logNine

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	0.70168275	0.07016827	42.46	<.0001
Error	16	0.02644340	0.00165271		
Corrected Total	26	0.72812615			

R-Square	Coeff Var	Root MSE	logNine Mean
0.963683	0.998590	0.040654	4.071099

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Block	2	0.00235613	0.00117806	0.71	0.5052
Treat	8	0.69932662	0.08741583	52.89	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Block	2	0.00235613	0.00117806	0.71	0.5052
Treat	8	0.69932662	0.08741583	52.89	<.0001

The GLM Procedure Dependent Variable: logTen

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	0.48123995	0.04812400	11.60	<.0001
Error	16	0.06640510	0.00415032		
Corrected Total	26	0.54764506			

R-Square	Coeff Var	Root MSE	logTen Mean
0.878744	1.514744	0.064423	4.253061

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Block	2	0.00067642	0.00033821	0.08	0.9221
Treat	8	0.48056354	0.06007044	14.47	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Block	2	0.00067642	0.00033821	0.08	0.9221
Treat	8	0.48056354	0.06007044	14.47	<.0001

Tukey's Studentized Range (HSD) Test for sqrTwo

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0.803054
Critical Value of Studentized Range	5.03101
Minimum Significant Difference	2.603

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Treat
A	1.8341	3	T5
A	1.7180	3	T2
A	1.7087	3	T1
A	1.6924	3	T3
A	1.6757	3	T6
A	1.6485	3	T8
A	1.6208	3	T9
A	1.5680	3	T7
A	1.3596	3	T4

Tukey's Studentized Range (HSD) Test for logThree

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0.037247
Critical Value of Studentized Range	5.03101
Minimum Significant Difference	0.5606

Means with the same letter are not significantly different.

Tukey Grouping		Mean	N	Treat
A		2.0888	3	T9
A		2.0542	3	T6
B	A	1.9423	3	T8
B	A	1.7572	3	T5
B	A	1.7457	3	T4
B	A	1.6816	3	T3
B	A	1.5443	3	T2
B		1.4692	3	T7
B		1.4398	3	T1

Tukey's Studentized Range (HSD) Test for logFour

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0.00321
Critical Value of Studentized Range	5.03101
Minimum Significant Difference	0.1646

Means with the same letter are not significantly different.

Tukey Grouping		Mean	N	Treat
A		2.85974	3	T9
	B	2.66689	3	T6
C	B	2.54381	3	T8
C		2.41740	3	T5
	D	2.23910	3	T3
	D	2.22519	3	T4
	D	2.18826	3	T2
	D	2.13840	3	T1
	D	2.09719	3	T7

Tukey's Studentized Range (HSD) Test for logFive

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0.002422
Critical Value of Studentized Range	5.03101
Minimum Significant Difference	0.1429

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Treat
A	3.29043	3	T9
B	3.02079	3	T8
B	3.00699	3	T6
B	2.88971	3	T5
C	2.70845	3	T4
C	2.65863	3	T2
C	2.61442	3	T3
C	2.59509	3	T7
C	2.59268	3	T1

Tukey's Studentized Range (HSD) Test for logSix

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0.005055
Critical Value of Studentized Range	5.03101
Minimum Significant Difference	0.2065

Means with the same letter are not significantly different.

Tukey Grouping		Mean	N	Treat
	A	3.55491	3	T9
B	A	3.35046	3	T8
B		3.29404	3	T5
B		3.28212	3	T6
	C	3.04720	3	T4
	C	3.03925	3	T7
	C	2.99499	3	T2
	C	2.95881	3	T3
	C	2.90873	3	T1

Tukey's Studentized Range (HSD) Test for logSeven

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0.009204
Critical Value of Studentized Range	5.03101
Minimum Significant Difference	0.2787

Means with the same letter are not significantly different.

Tukey Grouping		Mean	N	Treat
	A	3.85697	3	T9
B	A	3.67726	3	T6
B	A	3.63403	3	T8
B	C	3.57367	3	T5
D	C	3.32258	3	T2
D	C	3.31357	3	T7
D		3.28804	3	T3
D		3.27457	3	T4
D		3.25880	3	T1

Tukey's Studentized Range (HSD) Test for logEight

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0.003776
Critical Value of Studentized Range	5.03101
Minimum Significant Difference	0.1785

Means with the same letter are not significantly different.

Tukey Grouping		Mean	N	Treat
	A	4.24671	3	T9
B	A	4.09755	3	T6
B	C	4.06716	3	T8
	C	3.89592	3	T5
	D	3.67481	3	T4
	D	3.65629	3	T2
	D	3.64975	3	T7
	D	3.64875	3	T3
	D	3.62613	3	T1

Tukey's Studentized Range (HSD) Test for logNine

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0.001653
Critical Value of Studentized Range	5.03101
Minimum Significant Difference	0.1181

Means with the same letter are not significantly different.

Tukey Grouping			Mean	N	Treat
	A		4.33516	3	T9
	A		4.27103	3	T6
	A		4.25703	3	T8
	B		4.04047	3	T5
C	B		4.00544	3	T3
C	B	D	3.99679	3	T4
C	B	D	3.94069	3	T2
C		D	3.90623	3	T7
		D	3.88705	3	T1

Tukey's Studentized Range (HSD) Test for logTen

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0.00415
Critical Value of Studentized Range	5.03101
Minimum Significant Difference	0.1871

Means with the same letter are not significantly different.

Tukey Grouping			Mean	N	Treat
	A		4.50168	3	T9
B	A		4.39599	3	T6
B	A		4.38123	3	T8
B	C		4.26202	3	T5
	C		4.18816	3	T4
	C		4.15938	3	T2
	C		4.15156	3	T7
	C		4.15100	3	T3
	C		4.08652	3	T1

Appendix F: Key data analysis output for objective five

```
proc means data=Plant_height_trialOne mean std;
class treat;
var FIFTEEN_DAT THIRTY_DAT FORTYFIVE_DAT SIXTY_DAT;
run;

proc glm data=Plant_height_trialOne;
class block treat;
model FIFTEEN_DAT THIRTY_DAT FORTYFIVE_DAT SIXTY_DAT=block treat;
means treat/TUKEY;
run;
```

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The MEANS Procedure

Treat	Obs	Variable	Mean	Std Dev
T1	3	FIFTEEN_DAT	15.2000000	0.1000000
		THIRTY_DAT	31.3666667	1.0066446
		FORTYFIVE_DAT	58.8000000	0.7211103
		SIXTY_DAT	60.2333333	1.1239810
T2	3	FIFTEEN_DAT	14.8666667	0.6658328
		THIRTY_DAT	30.7666667	0.4163332
		FORTYFIVE_DAT	57.4666667	0.6429101
		SIXTY_DAT	58.4666667	1.3012814
T3	3	FIFTEEN_DAT	14.5000000	0.6244998
		THIRTY_DAT	30.1000000	0.7937254
		FORTYFIVE_DAT	57.7000000	0.4358899
		SIXTY_DAT	57.8666667	0.2309401
T4	3	FIFTEEN_DAT	15.1333333	0.3055050
		THIRTY_DAT	29.6000000	1.3527749
		FORTYFIVE_DAT	56.6666667	0.9073772
		SIXTY_DAT	57.2333333	0.7094599
T5	3	FIFTEEN_DAT	14.8333333	0.5507571
		THIRTY_DAT	28.9666667	0.3511885
		FORTYFIVE_DAT	56.1666667	1.2583057
		SIXTY_DAT	56.4666667	1.1239810
T6	3	FIFTEEN_DAT	14.5666667	0.2516611
		THIRTY_DAT	27.2666667	0.4041452
		FORTYFIVE_DAT	55.0000000	0.2645751
		SIXTY_DAT	55.3000000	0.2645751
T7	3	FIFTEEN_DAT	15.5333333	0.3214550
		THIRTY_DAT	31.1000000	0.7937254
		FORTYFIVE_DAT	57.1333333	0.4163332
		SIXTY_DAT	59.6666667	2.0207259
T8	3	FIFTEEN_DAT	15.0000000	0.2645751
		THIRTY_DAT	27.9333333	0.6027714
		FORTYFIVE_DAT	54.8666667	0.3214550
		SIXTY_DAT	55.1333333	1.8147543
T9	3	FIFTEEN_DAT	14.6000000	0.3605551
		THIRTY_DAT	25.6333333	1.0214369
		FORTYFIVE_DAT	50.3666667	0.7094599
		SIXTY_DAT	52.6666667	1.7097758

The GLM Procedure Dependent Variable: FIFTEEN_DAT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	3.10370370	0.31037037	1.73	0.1583
Error	16	2.87037037	0.17939815		
Corrected Total	26	5.97407407			

R-Square	Coeff Var	Root MSE	FIFTEEN_DAT Mean
0.519529	2.839822	0.423554	14.91481

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Block	2	0.34296296	0.17148148	0.96	0.4054
Treat	8	2.76074074	0.34509259	1.92	0.1263

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Block	2	0.34296296	0.17148148	0.96	0.4054
Treat	8	2.76074074	0.34509259	1.92	0.1263

The GLM Procedure Dependent Variable: THIRTY_DAT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	90.6170370	9.0617037	13.35	<.0001
Error	16	10.8614815	0.6788426		
Corrected Total	26	101.4785185			

R-Square	Coeff Var	Root MSE	THIRTY_DAT Mean
0.892968	2.822357	0.823919	29.19259

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Block	2	1.07851852	0.53925926	0.79	0.4689
Treat	8	89.53851852	11.19231481	16.49	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Block	2	1.07851852	0.53925926	0.79	0.4689
Treat	8	89.53851852	11.19231481	16.49	<.0001

The GLM Procedure Dependent Variable: FORTYFIVE_DAT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	146.2059259	14.6205926	27.47	<.0001
Error	16	8.5148148	0.5321759		
Corrected Total	26	154.7207407			

R-Square	Coeff Var	Root MSE	FORTYFIVE_DAT Mean
0.944967	1.302255	0.729504	56.01852

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Block	2	0.2451852	0.1225926	0.23	0.7968
Treat	8	145.9607407	18.2450926	34.28	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Block	2	0.2451852	0.1225926	0.23	0.7968
Treat	8	145.9607407	18.2450926	34.28	<.0001

The GLM Procedure Dependent Variable: SIXTY_DAT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	138.8770370	13.8877037	7.59	0.0002
Error	16	29.2925926	1.8307870		
Corrected Total	26	168.1696296			

R-Square	Coeff Var	Root MSE	SIXTY_DAT Mean
0.825815	2.373645	1.353066	57.00370

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Block	2	1.0007407	0.5003704	0.27	0.7643
Treat	8	137.8762963	17.2345370	9.41	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Block	2	1.0007407	0.5003704	0.27	0.7643
Treat	8	137.8762963	17.2345370	9.41	<.0001

Tukey's Studentized Range (HSD) Test for FIFTEEN_DAT

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	18
Error Mean Square	0.178519
Critical Value of Studentized Range	4.95521

Minimum Significant Difference 1.2088

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Treat
A	15.5333	3	T7
A	15.2000	3	T1
A	15.1333	3	T4
A	15.0000	3	T8
A	14.8667	3	T2
A	14.8333	3	T5
A	14.6000	3	T9
A	14.5667	3	T6
A	14.5000	3	T3

Tukey's Studentized Range (HSD) Test for THIRTY_DAT

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	18
Error Mean Square	0.663333
Critical Value of Studentized Range	4.95521
Minimum Significant Difference	2.3301

Means with the same letter are not significantly different.

Tukey Grouping			Mean	N	Treat
	A		31.3667	3	T1
B	A		31.1000	3	T7
B	A		30.7667	3	T2
B	A	C	30.1000	3	T3
B	A	C	29.6000	3	T4
B	D	C	28.9667	3	T5
E	D	C	27.9333	3	T8
E	D		27.2667	3	T6
E			25.6333	3	T9

Tukey's Studentized Range (HSD) Test for FORTYFIVE_DAT

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	18
Error Mean Square	0.486667
Critical Value of Studentized Range	4.95521
Minimum Significant Difference	1.9958

Means with the same letter are not significantly different.

Tukey Grouping		Mean	N	Treat
	A	58.8000	3	T1
B	A	57.7000	3	T3
B	A	57.4667	3	T2
B	A	57.1333	3	T7
B	C	56.6667	3	T4
B	C	56.1667	3	T5
	C	55.0000	3	T6
	C	54.8667	3	T8
	D	50.3667	3	T9

Tukey's Studentized Range (HSD) Test for SIXTY_DAT

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05

```
                Error Degrees of Freedom                 18
                Error Mean Square                   1.682963
                Critical Value of Studentized Range  4.95521
                Minimum Significant Difference        3.7114
        Means with the same letter are not significantly different.

              Tukey Grouping          Mean      N    Treat
                     A              60.233      3    T1
                B    A              59.667      3    T7
                B    A    C         58.467      3    T2
                B    A    C         57.867      3    T3
                B    A    C         57.233      3    T4
                B         C         56.467      3    T5
                     D    C         55.300      3    T6
                     D    C         55.133      3    T8
                     D              52.667      3    T9
```

```
proc means data= Stem_diameter_TrialOne mean std;
class treat;
var FifteenDAT ThirtyDAT FortyfiveDAT SixtyDAT;
run;

proc glm data= Stem_diameter_TrialOne;
class block treat;
model FifteenDAT ThirtyDAT FortyfiveDAT SixtyDAT=block treat;
means treat/TUKEY;
run;
```

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The MEANS Procedure

Treat	Obs	Variable	Mean	Std Dev
T1	3	FifteenDAT	4.2333333	0.2081666
		ThirtyDAT	6.5333333	1.0503968
		FortyfiveDAT	11.0333333	0.2886751
		SixtyDAT	14.4666667	0.1527525
T2	3	FifteenDAT	4.2333333	0.2081666
		ThirtyDAT	6.5333333	0.1527525
		FortyfiveDAT	10.8666667	0.2516611
		SixtyDAT	14.2666667	0.4509250
T3	3	FifteenDAT	4.3333333	0.1527525
		ThirtyDAT	6.6666667	0.4725816
		FortyfiveDAT	10.9666667	0.1527525
		SixtyDAT	13.7666667	0.6429101
T4	3	FifteenDAT	4.0666667	0.1527525
		ThirtyDAT	6.5000000	0.1000000
		FortyfiveDAT	11.4666667	0.8386497
		SixtyDAT	13.8333333	0.4041452
T5	3	FifteenDAT	3.9000000	0.1000000
		ThirtyDAT	6.3000000	0.4358899
		FortyfiveDAT	11.3000000	0.8717798
		SixtyDAT	13.5000000	0.4358899
T6	3	FifteenDAT	4.1333333	0.2309401
		ThirtyDAT	5.6333333	0.2081666
		FortyfiveDAT	11.6333333	0.2081666
		SixtyDAT	13.0666667	0.2081666
T7	3	FifteenDAT	4.1000000	0.1000000
		ThirtyDAT	6.8666667	1.4640128
		FortyfiveDAT	10.7000000	0.6928203

		SixtyDAT	14.4333333	0.3055050
T8	3	FifteenDAT	4.0333333	0.4041452
		ThirtyDAT	5.3666667	0.2081666
		FortyfiveDAT	11.4000000	0.8717798
		SixtyDAT	12.9333333	0.3055050
T9	3	FifteenDAT	3.9666667	0.3055050
		ThirtyDAT	5.1000000	0.2000000
		FortyfiveDAT	9.8666667	0.8326664
		SixtyDAT	12.6333333	0.4041452

Dependent Variable: FifteenDAT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	0.55555556	0.05555556	1.07	0.4364
Error	16	0.83111111	0.05194444		
Corrected Total	26	1.38666667			

R-Square	Coeff Var	Root MSE	FifteenDAT Mean
0.400641	5.543836	0.227913	4.111111

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Block	2	0.09555556	0.04777778	0.92	0.4187
Treat	8	0.46000000	0.05750000	1.11	0.4082

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Block	2	0.09555556	0.04777778	0.92	0.4187
Treat	8	0.46000000	0.05750000	1.11	0.4082

Dependent Variable: ThirtyDAT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	9.62888889	0.96288889	2.02	0.1006
Error	16	7.61111111	0.47569444		
Corrected Total	26	17.24000000			

R-Square	Coeff Var	Root MSE	ThirtyDAT Mean
0.558520	11.18442	0.689706	6.166667

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Block	2	0.02888889	0.01444444	0.03	0.9701
Treat	8	9.60000000	1.20000000	2.52	0.0548

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Block	2	0.02888889	0.01444444	0.03	0.9701
Treat	8	9.60000000	1.20000000	2.52	0.0548

Dependent Variable: FortyfiveDAT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	8.70592593	0.87059259	2.64	0.0408
Error	16	5.28592593	0.33037037		
Corrected Total	26	13.99185185			

R-Square	Coeff Var	Root MSE	FortyfiveDAT Mean
0.622214	5.212973	0.574779	11.02593

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Block	2	1.93407407	0.96703704	2.93	0.0825
Treat	8	6.77185185	0.84648148	2.56	0.0520

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Block	2	1.93407407	0.96703704	2.93	0.0825
Treat	8	6.77185185	0.84648148	2.56	0.0520

Dependent Variable: SixtyDAT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	10.92222222	1.09222222	6.46	0.0006
Error	16	2.70444444	0.16902778		
Corrected Total	26	13.62666667			

R-Square	Coeff Var	Root MSE	SixtyDAT Mean
0.801533	3.010715	0.411130	13.65556

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Block	2	0.06888889	0.03444444	0.20	0.8177
Treat	8	10.85333333	1.35666667	8.03	0.0002

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Block	2	0.06888889	0.03444444	0.20	0.8177
Treat	8	10.85333333	1.35666667	8.03	0.0002

Tukey's Studentized Range (HSD) Test for FortyfiveDAT

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0.33037
Critical Value of Studentized Range	5.03101
Minimum Significant Difference	1.6695

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Treat
A	11.6333	3	T6
B A	11.4667	3	T4
B A	11.4000	3	T8
B A	11.3000	3	T5
B A	11.0333	3	T1
B A	10.9667	3	T3
B A	10.8667	3	T2
B A	10.7000	3	T7
B	9.8667	3	T9

Tukey's Studentized Range (HSD) Test for SixtyDAT

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0.169028
Critical Value of Studentized Range	5.03101
Minimum Significant Difference	1.1942

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Treat
A	14.4667	3	T1
A	14.4333	3	T7
A	14.2667	3	T2
B A	13.8333	3	T4
B A C	13.7667	3	T3
B A C	13.5000	3	T5
B C	13.0667	3	T6
B C	12.9333	3	T8
C	12.6333	3	T9

```
proc glm data=YieldParameters_TrialOne;
class block treat;
model TruPlt FloTru logFrtTru    sqrNHltFrtplt sqrNboredFrtplt logYldHltFrtplt;
logYldBoredFrtplt = block treat;
means treat/Tukey;
run;
```

The MEANS Procedure

Treat	Obs	Variable	Mean	Std Dev
T1	3	TruPlt	11.8666667	0.1527525
		FloTru	9.6666667	0.4163332
		FrtTru	4.0666667	0.2516611
		NHltFrtplt	5.9333333	0.1527525
		Nboredfrtplt	5.8133333	0.2498666
		YldHltFrtplt	406.3000000	10.9000000
		YldBoredfrtplt	333.2666667	33.3672494
T2	3	TruPlt	11.5666667	0.3511885
		FloTru	8.5000000	0.1000000
		FrtTru	4.0333333	0.1154701
		NHltFrtplt	5.6333333	0.1527525
		Nboredfrtplt	5.1333333	0.3827967
		YldHltFrtplt	381.0000000	22.3138970
		YldBoredfrtplt	286.8333333	8.7688844
T3	3	TruPlt	11.7000000	0.2000000
		FloTru	7.6666667	0.1527525
		FrtTru	4.0666667	0.0577350
		NHltFrtplt	5.5000000	0.2645751
		Nboredfrtplt	6.0533333	0.4252450
		YldHltFrtplt	374.4333333	23.5058149
		YldBoredfrtplt	328.8333333	34.8993314
T4	3	TruPlt	11.3666667	0.1154701
		FloTru	7.3000000	0.3000000
		FrtTru	3.8333333	0.5033223
		NHltFrtplt	5.3666667	0.1527525
		Nboredfrtplt	5.5866667	0.3682843
		YldHltFrtplt	335.0000000	34.5483719
		YldBoredfrtplt	313.9333333	17.2818788
T5	3	TruPlt	10.9000000	0.2000000
		FloTru	7.4000000	0.1000000
		FrtTru	3.0000000	0.2645751
		NHltFrtplt	2.4666667	0.3055050
		Nboredfrtplt	3.1900000	0.1664332
		YldHltFrtplt	151.0333333	12.3216611
		YldBoredfrtplt	161.7333333	9.7109903
T6	3	TruPlt	10.8000000	0.3000000
		FloTru	6.4333333	0.2081666
		FrtTru	2.8666667	0.2081666
		NHltFrtplt	1.4666667	0.0577350
		Nboredfrtplt	2.5600000	0.2200000
		YldHltFrtplt	81.4666667	12.4708995
		YldBoredfrtplt	126.2333333	10.0857986
T7	3	TruPlt	12.0666667	0.0577350
		FloTru	9.4000000	0.5567764
		FrtTru	4.3000000	0.1732051
		NHltFrtplt	6.5000000	0.2000000
		Nboredfrtplt	4.8733333	0.5658033
		YldHltFrtplt	402.8666667	12.7032804
		YldBoredfrtplt	275.7333333	29.5434144
T8	3	TruPlt	10.8333333	0.3055050
		FloTru	6.5000000	0.2645751
		FrtTru	3.0000000	0.3605551
		NHltFrtplt	1.7333333	0.1527525
		Nboredfrtplt	2.3333333	0.5147168
		YldHltFrtplt	86.6000000	8.9604687
		YldBoredfrtplt	109.9000000	26.1917926
T9	3	TruPlt	10.8000000	0.5000000
		FloTru	5.5666667	0.6506407
		FrtTru	2.4666667	0.3055050

NHltFrtplt	0.7000000	0.4358899
Nboredfrtplt	1.9000000	0.5302829
YldHltFrtplt	32.5000000	22.8225765
YldBoredfrtplt	83.7333333	22.0663394

Dependent Variable: TruPlt

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	6.12222222	0.61222222	7.63	0.0002
Error	16	1.28444444	0.08027778		
Corrected Total	26	7.40666667			

R-Square	Coeff Var	Root MSE	TruPlt Mean
0.826583	2.502453	0.283333	11.32222

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Block	2	0.06888889	0.03444444	0.43	0.6584
Treat	8	6.05333333	0.75666667	9.43	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Block	2	0.06888889	0.03444444	0.43	0.6584
Treat	8	6.05333333	0.75666667	9.43	<.0001

Dependent Variable: FloTru

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	45.57703704	4.55770370	32.96	<.0001
Error	16	2.21259259	0.13828704		
Corrected Total	26	47.78962963			

R-Square	Coeff Var	Root MSE	FloTru Mean
0.953701	4.890638	0.371870	7.603704

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Block	2	0.09407407	0.04703704	0.34	0.7167
Treat	8	45.48296296	5.68537037	41.11	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Block	2	0.09407407	0.04703704	0.34	0.7167
Treat	8	45.48296296	5.68537037	41.11	<.0001

Dependent Variable: logFrtTru

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	1.02172594	0.10217259	16.53	<.0001
Error	16	0.09888524	0.00618033		
Corrected Total	26	1.12061117			

R-Square	Coeff Var	Root MSE	logFrtTru Mean
0.911758	6.355102	0.078615	1.237039

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Block	2	0.03693891	0.01846946	2.99	0.0789
Treat	8	0.98478702	0.12309838	19.92	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Block	2	0.03693891	0.01846946	2.99	0.0789
Treat	8	0.98478702	0.12309838	19.92	<.0001

Dependent Variable: sqrNHltFrtplt

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	10.28968634	1.02896863	150.79	<.0001
Error	16	0.10917988	0.00682374		
Corrected Total	26	10.39886621			

R-Square	Coeff Var	Root MSE	sqrNHltFrtplt Mean
0.989501	4.392269	0.082606	1.880712

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Block	2	0.05655614	0.02827807	4.14	0.0355

Treat	8	10.23313020	1.27914127	187.45	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Block	2	0.05655614	0.02827807	4.14	0.0355
Treat	8	10.23313020	1.27914127	187.45	<.0001

Dependent Variable: sqrNboredFrtplt

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	4.38559299	0.43855930	33.17	<.0001
Error	16	0.21152634	0.01322040		
Corrected Total	26	4.59711933			

R-Square	Coeff Var	Root MSE	sqrNboredFrtplt Mean
0.953987	5.756122	0.114980	1.997525

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Block	2	0.01458374	0.00729187	0.55	0.5866
Treat	8	4.37100925	0.54637616	41.33	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Block	2	0.01458374	0.00729187	0.55	0.5866
Treat	8	4.37100925	0.54637616	41.33	<.0001

Dependent Variable: logYldHltFrtplt

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	22.57966523	2.25796652	47.14	<.0001
Error	16	0.76645630	0.04790352		
Corrected Total	26	23.34612153			

R-Square	Coeff Var	Root MSE	logYldHltFrtplt Mean
0.967170	4.201962	0.218869	5.208726

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Block	2	0.19018504	0.09509252	1.99	0.1698
Treat	8	22.38948018	2.79868502	58.42	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Block	2	0.19018504	0.09509252	1.99	0.1698
Treat	8	22.38948018	2.79868502	58.42	<.0001

Dependent Variable: logYldBoredFrtplt

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	7.14221034	0.71422103	34.28	<.0001
Error	16	0.33332049	0.02083253		
Corrected Total	26	7.47553084			

R-Square	Coeff Var	Root MSE	logYldBoredFrtplt Mean
0.955412	2.727605	0.144335	5.291631

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Block	2	0.02136888	0.01068444	0.51	0.6083
Treat	8	7.12084146	0.89010518	42.73	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Block	2	0.02136888	0.01068444	0.51	0.6083
Treat	8	7.12084146	0.89010518	42.73	<.0001

Tukey's Studentized Range (HSD) Test for TruPlt

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0.080278
Critical Value of Studentized Range	5.03101
Minimum Significant Difference	0.823

Means with the same letter are not significantly different.

Tukey Grouping			Mean	N	Treat
	A		12.0667	3	T7
	A		11.8667	3	T1
B	A		11.7000	3	T3
B	A	C	11.5667	3	T2
B	A	C	11.3667	3	T4
B		C	10.9000	3	T5
		C	10.8333	3	T8
		C	10.8000	3	T6
		C	10.8000	3	T9

Tukey's Studentized Range (HSD) Test for FloTru

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0.138287
Critical Value of Studentized Range	5.03101
Minimum Significant Difference	1.0802

Means with the same letter are not significantly different.

Tukey Grouping			Mean	N	Treat
		A	9.6667	3	T1
	B	A	9.4000	3	T7
	B	C	8.5000	3	T2
	D	C	7.6667	3	T3
	D	E	7.4000	3	T5
	D	E	7.3000	3	T4
	F	E	6.5000	3	T8
	F	E	6.4333	3	T6
	F		5.5667	3	T9

Tukey's Studentized Range (HSD) Test for logFrtTru

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0.00618
Critical Value of Studentized Range	5.03101
Minimum Significant Difference	0.2283

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Treat
A	1.45808	3	T7
A	1.40276	3	T3
A	1.40153	3	T1
A	1.39432	3	T2
A	1.33784	3	T4
B	1.09593	3	T5
B	1.09361	3	T8
B	1.05142	3	T6
B	0.89785	3	T9

Tukey's Studentized Range (HSD) Test for sqrNHltFrtplt

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0.006824
Critical Value of Studentized Range	5.03101
Minimum Significant Difference	0.2399

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Treat
A	2.54931	3	T7
A	2.43571	3	T1
A	2.37332	3	T2
A	2.34475	3	T3
A	2.31645	3	T4
B	1.56858	3	T5
C	1.31572	3	T8
C	1.21090	3	T6
D	0.81167	3	T9

Tukey's Studentized Range (HSD) Test for sqrNboredFrtplt

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0.01322
Critical Value of Studentized Range	5.03101
Minimum Significant Difference	0.334

Means with the same letter are not significantly different.

Tukey Grouping		Mean	N	Treat
A		2.45934	3	T3
A		2.41071	3	T1
A		2.36276	3	T4
A		2.26464	3	T2
A		2.20513	3	T7
	B	1.78565	3	T5
C	B	1.59901	3	T6
C	B	1.52083	3	T8
C		1.36966	3	T9

Tukey's Studentized Range (HSD) Test for logYldHltFrtplt

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Error Degrees of Freedom	16
Error Mean Square	0.047904
Critical Value of Studentized Range	5.03101
Minimum Significant Difference	0.6357

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Treat
A	6.0069	3	T1
A	5.9983	3	T7
A	5.9416	3	T2
A	5.9241	3	T3
A	5.8105	3	T4
B	5.0153	3	T5
B	4.4578	3	T8
B	4.3927	3	T6
C	3.3313	3	T9

Tukey's Studentized Range (HSD) Test for logYldBoredFrtplt

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0.020833
Critical Value of Studentized Range	5.03101
Minimum Significant Difference	0.4192

Means with the same letter are not significantly different.

Tukey Grouping		Mean	N	Treat
A		5.8057	3	T1
A		5.7919	3	T3
A		5.7482	3	T4
A		5.6586	3	T2
A		5.6154	3	T7
B		5.0848	3	T5
B		4.8359	3	T6
C	B	4.6783	3	T8
C		4.4059	3	T9

Appendix G: Published paper on objective one

Ndereyimana *et al. Egyptian Journal of Biological Pest Control* (2019) 29:57
https://doi.org/10.1186/s41938-019-0163-3

Egyptian Journal of Biological Pest Control

RESEARCH Open Access

Potential of entomopathogenic nematode isolates from Rwanda to control the tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae)

Assinapol Ndereyimana[1,2*], Samuel Nyalala[1], Patrick Murerwa[1] and Svetlana Gaidashova[2]

Abstract

Tomato leaf miner, *Tuta absoluta* (Meyrick), is a major threat to tomato production as it can cause up to 100% yield loss under both greenhouse and open-field conditions. Chemical control, which is associated with several undesirable effects, remains the only option readily available for this pest since its invasion of Rwanda in the year 2015. This study assessed the potential of using local isolates of entomopathogenic nematodes (EPNs) in management of *T. absoluta* in Rwanda. Six EPNs including four locally isolated strains: *Steinernema* sp. RW14-M-C2a-3, *Steinernema* sp. RW14-M-C2b-1, *S. carpocapsae* RW14-G-R3a-2 and *Heterorhabditis bacteriophora* RW14-N-C4a, and two exotic species: *S. carpocapsae* All and *H. bacteriophora* H06 were evaluated. Three bioassays were conducted in the laboratory, using a tomato leaflet with third instar *T. absoluta* larva in gallery and 9-cm Petri dishes as bioassay arenas in a completely randomized design with three replications. The EPNs were applied at a volume of 1 ml containing 500 infective juveniles per leaflet, while sterile tap water was used as negative control. Larval mortality was checked continuously for 96 h at 24 h interval. The results revealed that all the tested EPNs were able to find and kill *T. absoluta* larvae inside the leaf galleries; and their efficacy increased with exposure time. The pathogenicity effects were significantly different ($p < 0.05$) among EPNs. In the first 24 h after inoculation, the efficacy of local EPN isolates (53.3–96.7%) was significantly higher than the one of exotic species (0.0–26.7%). The efficacy of three Rwandan EPN isolates, *Steinernema* sp. RW14-M-C2a-3, *Steinernema* sp. RW14-M-C2b-1, and *S. carpocapsae* RW14-G-R3a-2 was not significantly different from 24 to 96 h after inoculation, except for *S. carpocapsae* RW14-G-R3a-2 during 24 h after inoculation in bioassay 3. There was insignificant difference among all the EPN isolates after 96 h of exposure. This is the first study carried out in Rwanda that investigated the potential of locally isolated EPNs against *T. absoluta*. Field experiments should be conducted to fully explore the possibilities of using local EPN isolates in integrated pest management of *T. absoluta* in Rwanda.

Keywords: *Tuta absoluta*, Entomopathogenic nematodes, *Heterorhabditis*, *Steinernema*, Local isolates, Biological control, Rwanda

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Appendix H: Published paper on objective two

Ndereyimana *et al. Egyptian Journal of Biological Pest Control* (2019) 29:70
https://doi.org/10.1186/s41938-019-0184-y

Egyptian Journal of Biological Pest Control

RESEARCH Open Access

Pathogenicity of some commercial formulations of entomopathogenic fungi on the tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae)

Assinapol Ndereyimana[1,2*], Samuel Nyalala[1], Patrick Murerwa[1] and Svetlana Gaidashova[2]

Abstract

The tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), is a major threat to tomato production in Rwanda. Laboratory bioassays were conducted to evaluate some commercial entomopathogenic fungi (EPF) formulations on *T. absoluta* larvae. The larvae, inside the leaf galleries, were obtained from the established tomato field. Commercial EPF: Metatech® WP [*Metarhizium anisopliae* (Metschn.) Sorok, Strain FCM Ar 23B3], Beauvitech® WP [*Beauveria bassiana* (Bals.) Vuill., Strain J25], and Botanigard ES [*B. bassiana* (Bals.), Strain GHA] were tested in Petri dishes against *T. absoluta* larvae at a concentration of 10^8 spores/ml. A synthetic insecticide, imidacloprid was included for comparison as a positive control, while water was used as a negative control. All the tested commercial EPF formulations were pathogenic to *T. absoluta* larvae in all conducted bioassays. Mortality rates increased with an increase in time (days). However, the insignificant difference was observed in the mortality of *T. absoluta* larvae treated with the commercial EPF during the first 3 days in all bioassays. Highly significant differences ($p < 0.01$) in pathogenicity among treatments were observed from the 4th to 6th days after inoculation. Metatech® WP and Beauvitech® WP recorded the highest mortality rates (82.8 and 60.8%) with the LT_{50} values of 3.9 and 5.2 days, respectively, while imidacloprid caused the least larval mortality. Since the EPF demonstrated high virulence level against the target pest, the efficacy of Metatech® WP and Beauvitech® WP should be advanced to field evaluation to determine their potential as alternatives to the synthetic insecticides.

Keywords: *Tuta absoluta*, Entomopathogenic fungi, *Beauveria bassiana*, *Metarhizium anisopliae*, Biological control, Rwanda

Background

Despite its economic and nutritional importance, tomato production in Rwanda is challenged by various factors, including the prevalence of pests and diseases, limited skills in pest and disease management, as well as lack of appropriate pest management options (Clay and Turatsinze 2014). The tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), is a major threat to tomato production in Rwanda since the year 2015 (FAO 2015). It damages the plant by mining the leaves and boring into the stems and fruits, resulting in reduced tomato yield and fruit quality (Brévault et al. 2014). The mines and holes inflicted by this pest also serve as entry points for secondary infectious pathogens. Tomato production losses reached up to 100% due to this pest (Desneux et al. 2010).

So far, little has been done to develop an integrated pest management (IPM) program fitting to Rwandan conditions for this pest. *T. absoluta* control in Rwanda is mainly based on chemical insecticides; which affects the populations of beneficial organisms, especially pollinators and natural enemies and causes water pollution and disturbance of aquatic ecosystems as well as human health problems (Shalaby et al. 2013). Furthermore, the continuous use of chemical pesticides has been reported

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Appendix I: Published paper on objective three

Journal of Applied Horticulture, 21(2), 2019

Bioactivity of plant extracts against tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae)

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Abstract

Tomato (*Solanum lycopersicum* L.) is economically and nutritionally important in Rwanda but its production is challenged by the tomato leaf miner (*Tuta absoluta* Meyrick), an invasive pest. Synthetic insecticides which are the main method of its control have various drawbacks. Bioactivity of *Tephrosia vogelii*, *Tithonia diversifolia*, *Vernonia amygdalina* and *Phytolacca dodecandra* acqueous extracts was evaluated against *T. absoluta* in laboratory. Leaflets with third instar larvae (3.85 - 5.65 mm) of *T. absoluta* in mines were collected from established tomato field. Aqueous plants extracts were evaluated at a dose of 10 % weight/volume. Sterile tap water and azadirachtin 0.03 % EC were used as negative and positive controls, respectively. Petri-dishes of 9 cm diametre (n=10) were used as bioassay arenas in a completely randomized design with four replications. Data on larvae mortality were collected every 24 h for a period of 5 days. Three bioassays were conducted on different dates. Results indicated that tested plant extracts exhibited a capacity to kill *T. absoluta* larvae in tomato leaf galleries with significant difference among them ($P < 0.0001$). The killing capacity increased with exposure time. At 24 h of exposure, *T. absoluta* larvae mortality was in a range of 35.0 - 37.5 % for azadirachtin and 5.0 - 10.0 % for *T. vogelii* while all other aqueous extracts had 0.0 % mortality, except *V. amygdalina* which recorded 2.5 % in bioassay one. In all bioassays, the lowest mortality recorded 5 days after treatments with *T. vogelii*, *T. diversifolia, V. amygdalina, P. dodecandra* and azadirachtin was 32.2, 2.8. 2.5, 20.5 and 97.5 % while the highest mortality at this time was 35.1, 10.6, 13.3, 24.9 and 100 %, respectively. *Tephrosia vogelii and P. dodecandra* which recorded higher efficacy compared to the other local plants should be advanced to field evaluation. The observed higher efficacy of azadirachtin to Rwandan population of *T. absoluta* should also be confirmed under field conditions.

Key words: Biopesticides, Botanicals, Insecticidal plants, *Phytolacca dodecandra*, *Solanum lycopersicum* L., *Tephrosia vogelii, Tithonia diversifolia*, *Vernonia amygdalina*

Introduction

Insect pests are one of the important causes for crop production losses all-over the world (Silva *et al*., 2011; Biondi *et al*., 2018). In particular, tomato leaf miner (*Tuta absoluta*), an invasive pest reported in Rwanda in 2015 (Uzayisenga *et al*., 2016) causes serious damage to tomato crop resulting in severe yield losses up to 100 % (Desneux *et al*., 2010). The pest is now spread in all tomato production areas of Rwanda (Uzayisenga *et al*., 2016). Thus, it is vital to develop effective management strategies against this challenging pest.

Management of insect pests is crucial to ensure good crop productivity. Use of synthetic insecticides, the main method of insect control all-over the world (Senthil-Nathan, 2013), often results in pollution of ecosystems, apparition of resistant pest genotypes and new pests and destruction of natural enemies among others (Macharia *et al*., 2009; Yalçin *et al*., 2015). Fortunately, there are various plants that possess different chemicals recognised as secondary metabolites which have insecticidal properties and hence the potential of being used to manage various insect pests (Adeyemi, 2010; Shrivastava and Singh, 2014).

Research on botanical insecticides have been carried out for many years with the main goal to minimize the harmful effects of synthetic insecticides (Adeyemi, 2010). Azadirachtin is one of the widely known and successful example of botanical insecticide discovery from plants (Mordue and Alasdair, 2000). It is effective against several pests and comparatively harmless to natural enemies than most of the commonly used synthetic insecticides (Gontijo *et al*., 2015). El-ghany *et al*. (2016) obtained up to 92 % *T. absoluta* larval mortality caused by azadirachtin. Tomé *et al*. (2013) also observed that azadirachtin is effective against *T. absoluta*. Although high efficacy was obtained with insecticides of plant origin like azadirachtin (Yalçin *et al*., 2015), they are relatively expensive. Therefore, evaluation and exploitation of extracts of locally available plants against *T. absoluta* is necessary because they are cheap, easy to prepare and contain multiple active components which impede the development of insect resistance (Braham *et al*., 2012).

Over two thousand plant species are reported to have insecticidal properties (Shivakumar *et al*., 2013) and studies have shown higher bioactivity of extracts from some plants such as *Acmella oleracea* (Asteraceae) *and Thymus vulgaris* (Lamiaceae) against *T. absoluta* larvae (Moreno *et al*., 2012; Nilahyane *et al*., 2012). Screening different plants to assess their potential against insect pests, including *T. absoluta*, would contribute to sustainable pest management while preserving environment.

Locally available plant materials, such as *Tephrosia vogelii*

Appendix J: Published paper on objective four

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Field efficacy of entomopathogens and plant extracts on *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae) infesting tomato in Rwanda

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ABSTRACT

Following its outbreak in 2015, *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae) immediately became one of the major threats to the food chain in Rwanda and, therefore, sustainable management options are needed to address the situation. Two field trials were established on 3rd April and 28th June 2019 to study the efficacy of entomopathogens and plant extracts on *T. absoluta* infestation in Rwanda. Similar procedures were followed and nine treatments were evaluated, including: entomopathogenic nematodes (EPNs) (*Steinernema* sp. RW14-M-C2a-3 and *Steinernema* sp. RW14-M-C2a-3), commercial formulations of entomopathogenic fungi (EPFs) [Metatech®WP: *Metarhizium anisopliae* (Metsch.) Sorok, Strain FCM Ar 23B3), Beauvitech® WP: *Beauveria bassiana* (Bals.) Vuill., Strain J2S], plant extracts of *Tephrosia vogelii* and *Phytolacca dodecandra*, azadirachtin 0.03% EC, imidacloprid as positive control and water as negative control. The entomopathogens and azadirachtin significantly reduced leaf and leaflet damages compared to the plant extracts and the controls. However, leaf damage increased with time and reached the maximum level (100%) in 9–10 weeks after transplanting in all the treatments. In both trials, the maximum leaflet damage observed with entomopathogens and azadirachtin in 10 weeks after transplanting varied between 59.7% and 74.7% with the marketable fruit yield of 12.4–16.2 t ha^{-1}; while leaflet damage in positive control ranged 80.0%–92.1% with marketable yield of 3.0–3.5 t ha^{-1}. Our results suggest that the entomopathogens and azadirachtin have the potential for use in integrated pest management of *T. absoluta* in Rwanda, but further studies are needed to incorporate them in the IPM program.

1. Introduction

Control of pests is a pre-requisite for enhanced crop performance and subsequent production as they can inflict severe damage resulting in total destruction (Desneux et al., 2010). Specifically, the tomato leaf miner, *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae) is a major challenge to tomato (*Solanum lycopersicum* L.) production in many parts of the world (Biondi et al., 2018). Following its outbreaks in Rwanda in 2015, FAO (2015) declared this pest among the most important threat.

Tuta absoluta larvae damage all parts of tomato plants, including stems, leaves, flowers, and fruits, resulting in interrupted crop growth and development (Biondi et al., 2018). By feeding within the mesophyll, one larva can make many galleries, moving in and out the leaves (Gözel and Kasap, 2015). Studies reported a positive correlation between leaf and fruit infestations (Cocco et al., 2014). Up to 12 generations of this pest are possible under favourable conditions, which add to its invasive nature (Biondi et al., 2018). In the absence of proper management measures, yield losses inflicted by this pest can reach 100% of the total production (Desneux et al., 2010). Chemical control is the main option used by most African farmers to manage this pest. However, *T. absoluta* management remains a challenge mainly due to its mine-feeding habit, short development cycle, and acquisition of resistance to frequently used pesticides (Roditakis et al., 2013, 2015). This, in addition to the harmful effects of chemicals in the environment, necessitates the need for sustainable alternatives.

Sex pheromones have been widely and successfully used to detect and monitor the population of *T. absoluta* (Megido et al., 2013). In addition pheromone traps have been recommended and used for mass trapping and mating disruption of *T. absoluta* males (Witzgall et al., 2010; Harbi et al., 2012). At high infestation, mating disruption

Abbreviations: EPNs, Entomopathogenic nematodes; EPFs, Entomopathogenic fungi; PEs, Plant extracts; IJs, Infective juveniles.

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Appendix K: Published paper on objective five

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AHS
Advances in Horticultural Science

Growth, yield and fruit quality of tomato under different integrated management options against *Tuta absoluta* Meyrick

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Key words: azadirachtin, *Beauveria bassiana*, integrated pest management, *Metarhizium anisopliae*, *Phytolacca dodecandra*, *Solanum lycopersicum* L., *Steinernema*, *Tephrosia vogelii*.

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Data Availability Statement:
All relevant data are within the paper and its Supporting Information files.

Competing Interests:
The authors declare no competing interests.

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Abstract: This study evaluated the effect of entomopathogens and plant extracts, used against *Tuta absoluta*, on growth, yield, and fruit quality of tomato. Two field trials were carried out in a randomised compled block design, replicated thrice. The treatments were *Steinernema* sp. RW14-M-C2a-3, *Steinernema* sp. RW14-M-C2a-3, Metatech®WP (*Metarhizium anisopliae*, Strain FCM Ar 23B3), Beauvitech®WP (*Beauveria bassiana*, Strain J25) as entomopathogens, *Tephrosia vogelii* and *Phytolacca dodecandra* as plant extracts, and azadirachtin 0.03% EC. Imidacloprid and water also were included as positive and negative controls, respectively. The best growth and yield parameters were recorded with the entomopathogens and azadirachtin, which were insignificantly different in most cases. The increase in yied of healthy fruit per plant (average of two trials) compared to the negative control (water spray) was 11.4, 10.8,10.1, 9.6, 3.96, 2.2, 11.7 and 2.4 folds for *Steinernema* sp. RW14-M-C2a-3, *Steinernema* sp. RW14-M-C2a-3, Metatech®WP, Beauvitech WP, *T. vogelii, P. dodecandra*, azadirachtin, and imidacloprid, respectively. There was no significant difference in number of leaves per plant and fruit quality parameters. The entomopathogens and azadirachtin, which exhibited a capacity to enhance tomato growth and reduced yield losses due to *T. absoluta*, are recommended to be included in integrated pest management programme on tomato.

1. Introduction

The increasing world population requires food security, which can be partly achieved by reducing the portion of food lost every year as a result of pests (Kumar and Omkar, 2018). However, yield losses inflicted by crop pests have been observed to increase constantly despite different strategies being implemented globally (Dhaliwal *et al.*, 2010).

Tomato (*Solanum lycopersicum* L.) is one of the most popular vegeta-

Appendix L: Poster presentation on objective one at African Potato Association conference

Potential of entomopathogenic nematode isolates from Rwanda to control tomato leaf miner, *Tuta absoluta* (Meyrick 1917)

Asinapol NDEREYIMANA[1,2], Samuel NYALALA[1], Patrick MURERWA[1] Svetlana GAIDASHOVA[2]
[1]Rwanda Agriculture Board, [2]Egerton University

Abstract

Tuta absoluta is a new pest of tomato crop in Rwanda since the year 2015 and can cause up to 100 % loss of total tomato yield. It has also been reported on potato (*Solanum tuberosum* L.). Chemical control is associated with several drawbacks (Macharia *et al.*, 2009). The potential of using local isolates of entomopathogenic nematodes (EPNs) in management of *T. absoluta* in Rwanda was assessed. Three bioassays were conducted in Biological control laboratory – EPN production facility of Rwanda Agriculture and Animal Resources Development Board (RAB). Six EPNs including 4 locally isolated strains and 2 exotic species EPNs were applied at a volume of 1 ml containing 500 infective juveniles per leaflet having larva of *T. absoluta* inside the mine. Sterile tap water was used as negative control. Larval mortality was checked for 96 h with 24 h interval. All tested EPNs were able to find and kill *T. absoluta* larvae inside the leaf galleries; their efficacy increased with time and was significantly different ($p < 0.05$) among EPNs. There was no significant difference among all EPN isolates after 96 h of exposure and 100 % mortality was observed in most cases. Field evaluation should be the next step.

Figure 1. Damage caused by *Tuta absoluta* on tomato fruit and leaf

Introduction + Study objective

- *Tuta absoluta* Meyrick 1917) is a major threat to tomato production in Rwanda since 2015 (FAO, 2015).
- It can cause up to 100 % loss of total tomato yield (Biondi *et al.*, 2018).
- It has also been reported on other *Solanaceae* crops, including potato (*Solanum tuberosum* L.).
- Chemical control remains the only option but it is associated with several drawbacks (Macharia *et al.*, 2009).
- The potential of using local isolates of entomopathogenic nematodes (EPNs) in management of *T. absoluta* in Rwanda was assessed.

Material and methods

- **Area of study**: Biological Control Laboratory - EPN Production Facility of Rwanda Agriculture and Animal Resources Development Board.
- **Period of study**: January – February, 2019

Table1. Evaluated entomopathogenic nematodes against *Tuta absoluta*

EPN isolates	Source
Steinernema sp. RW14-M-C2a-3	Locally isolated species (Rwanda)
Steinernema sp. RW14-M-C2b-1	
S. carpocapsae RW14-G-R3a-2	
Heterorhabditis bacteriophora RW14-N-C4a	
S. carpocapsae All	Exotic species (China)
H. bacteriophora H06	

Material and methods

- Three bioassays were conducted in laboratory,
- Experimental design: completely randomized design with 3 replications,
- Petri-dishes (9 cm diameter) were as bioassay arenas,
- Tomato leaflet, with third instar *T. absoluta* larva in gallery, was put in each Petri-dish,
- EPNs were applied at a volume of 1 ml containing 500 infective juveniles per leaflet,
- Sterile tap water was used as negative control,
- Larval mortality was checked for 96 h with 24 h interval.

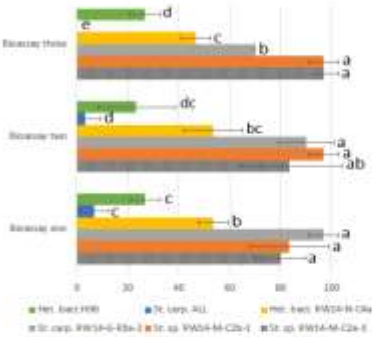

Figure 2. Mortality % of *Tuta absoluta* larvae (mean ± SD) in leaf galleries, 24h after treatment with local and exotics EPNs using the concentration of 500 Ils/ml. St=Steinernema; Het=Heterorhabditis; St carp=Steinernema carpocapsae; Het bact=Heterorhabditis bacteriophora; Mean followed by different letters in within the same bioassay are significantly different according to Tukey's test ($P < 0.05$).

Results and Discussion

- All tested EPNs were able to find and kill *T. absoluta* larvae inside the leaf galleries and their efficacy increased with time.
- The pathogenicity effects were significantly different ($p < 0.05$) among EPNs.
- In the first 24 h after inoculation, the efficacy of local EPN isolates (53.3 - 96.7 %) was significantly higher than the exotic species (0.0 - 26.7 %).
- The efficacy of three Rwandan EPN isolates, *Steinernema* sp. RW14-M-C2a-3, *Steinernema* sp. RW14-M-C2b-1 and *S. carpocapsae* RW14-G-R3a-2 remained not significantly different from 24 to 96 h after inoculation.
- There was no significant difference among all EPN isolates after 96 h of exposure and 100 % mortality was observed in most cases.

Conclusions

- This is the first study carried out in Rwanda that investigated the potential of locally isolated EPNs against *T. absoluta*.
- Field experiments will help to fully explore the possibility of using local EPN isolates in Integrated Pest Management of *T. absoluta* in Rwanda

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United States Agency for International Development, Rwanda Agriculture Board, Egerton University

APA AFRICAN POTATO ASSOCIATION

Poster presented on APA Conference 25-29 August 2019 Kigali Rwanda

Appendix M: Poster presentation on objective two at African Potato Association conference

Pathogenicity of selected commercial entomopathogenic fungal formulations on tomato leaf miner, *Tuta absoluta* Meyrick

Assinapol NDEREYIMANA[1,2], Samuel MWALILA[2], Patrick MUREBWA[1] Svetlana GADDANKOVA[2]
[1]Rwanda Agriculture Board, [2]Egerton University

Abstract

Laboratory bioassays were conducted to evaluate selected commercial entomopathogenic fungi (EPF) formulations on tomato leaf miner (*Tuta absoluta*), a major threat to tomato production in Rwanda. *Tuta absoluta* larvae inside the leaf galleries were obtained from established tomato field. Three commercial EPF were tested in petri-dish (n=10) bioassays at a concentration of 10^8 spores ml^{-1} in a completely randomized experiment with four replications. A synthetic insecticide, imidacloprid and sterilized tap water were included as positive and negative controls, respectively. Larval mortality was checked every 24 h for a period of 144 h (6 days). The whole bioassay was repeated three times. In all bioassays, no significant difference was observed in mortality of *T. absoluta* larvae treated with the commercial EPF during the first three days. From fourth to sixth day, larval mortality increased gradually with Metatech® WP and Beauvitech® WP recording the highest mortality of 82.8 and 60.8 % with LT_{50} of 3.9 and 5.2 days, respectively.

Introduction + Study objective

- Invasive species are a major threat to food production.
- Tomato leaf miner, *Tuta absoluta* Meyrick, invaded Rwanda in the year 2015 (FAO, 2015), and it is able to cause serious damage to tomato crop and reduce dramatically its yield (Desneux *et al.*, 2010).
- It has also been reported on other Solanaceae crops like potato (*Solanum tuberosum* L.), eggplant (*Solanum melongena* L.) and pepper (*Capsicum annuum* L.).
- In order to reduce dependence upon chemical insecticides, the development of alternative measures should be given a particular emphasis.
- Laboratory bioassays were conducted to evaluate selected commercial entomopathogenic fungi (EPF) formulations against *T. absoluta*

Figure 1. Commercial entomopathogenic fungi formulations evaluated against *Tuta absoluta*

Figure 2. Observation of viability of *Tuta absoluta* larvae before application of treatments

Material and methods

- **Area of study:** Biological Control Laboratory - EPN Production Facility of Rwanda Agriculture and Animal Resources Development Board.
- **Period of study:** November – December, 2018
- **Commercial EPF evaluated:** Metatech®WP (*Metarhizium anisopliae*, Strain FCM Ar 23B3), Beauvitech® WP (*Beauveria bassiana*, Strain I25) and Botanigard ES (*B. bassiana*, strain GHA).
- Concentration used: 10^8 spores ml^{-1}
- Imidacloprid and sterilized tap water were included as positive and negative controls, respectively. Larval mortality was checked every 24 h for a period of 144 h (6 days).
- Experimental design: CRD with 4 replications. Number of bioassays: 3.
- The effect of treatments on *T. absoluta* larvae mortality was evaluated using GLM procedure.
- Means for statistically different treatments were separated using Tukey's honestly significant difference (HSD) test at 5 % level of significance.
- Lethal time to kill 50 % of treated larvae (LT_{50}) was computed for all evaluated EPF formulations through probit analysis
- Statistical Analysis System package SAS software version 9.2 was used for data analysis.

Results and Discussion

- In all bioassays, no significant difference was observed in mortality of *T. absoluta* larvae treated with the commercial EPF during the first three days.
- From fourth to sixth day, larval mortality increased gradually with Metatech® WP and Beauvitech® WP recording the highest mortality of 82.8 and 60.8 % with LT_{50} of 3.9 and 5.2 days, respectively.
- The efficacy of Metatech® WP and Beauvitech® WP should be advanced to field evaluation stage to determine their potential as alternatives to synthetic insecticides.

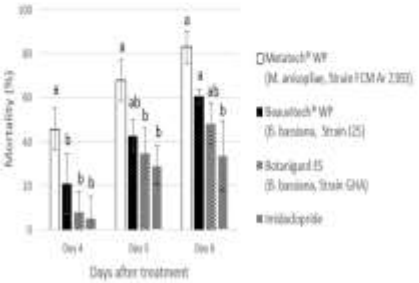

Figure 3. Mortality (%) of *Tuta absoluta* larvae treated with commercial EPF formulations (10^8 spores ml^{-1}) and imidacloprid (control) in bioassays three. Different letters above bars (Mean + SD) within the same day indicate significant difference according to Tukey test ($p < 0.05$).

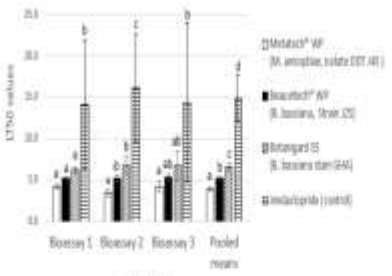

Figure 4. Lethal time (days) to kill 50 % of *Tuta absoluta* larvae treated with commercial EPF formulations (10^8 spores ml^{-1}) and imidacloprid (1 ml L^{-1}). Different letters above the means + SD bars indicate significant difference within the same bioassay according to Tukey test ($p < 0.05$).

Conclusions

- Since the ultimate entomopathogenic agent must demonstrate high virulence level against the target pest, **Metatech® WP and Beauvitech® WP** should be the first ones to be advanced to field evaluation stage to further determine their potential as alternative to chemical insecticides.
- Since EPF mass production is cost effective, they have substantial advantages of being used in biological control of *T. absoluta*.

References

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APA AFRICAN POTATO ASSOCIATION
Poster presented on APA Conference 25-29 August 2019 Kigali Rwanda

Appendix N: Research Permit

REPUBLIC OF RWANDA
RWANDA AGRICULTURE AND ANIMAL
RESOURCES DEVELOPMENT BOARD(RAB)
Office of the Director General
HUYE-RWANDA

Huye, on..29.../..01..../ 2020
Ref: N° 01.11/153.. /020/PK/HQ

The Director
Board of Postgraduates Studies
Egerton University
P.O. Box 536-20115
Egerton, Njoro,
KENYA

Dear Madam,

RE : Autorisation and assistance rendered to Mr. Assinapol NDEREYIMANA in regard to his PhD Research activities

Reference is made to your letter N°. KD14/13012/17 dated 29th June, 2018 introducing Mr. Assinapol NDEREYIMANA (Researcher at Rwanda Agriculture and Animal Resources Development Board-RAB, enrolled as a *bona fide* PhD student in Horticulture at Egerton University), in regard to his field research activities on "**Development of Options for Integrated Pest Management of *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae) for Enhanced Tomato Productivity in Rwanda;"**

I am writing to confirm that Mr. Assinapol NDEREYIMANA has been authorized to conduct his field and laboratory research activities at RAB from September 2018 to December 2019. He has been facilitated in acquisition of import permits for introduction of commercial formulations of entomopathogenic fungi which were used during his study, and was allowed to access and use entomopathogenic nematodes (EPNs) maintained in the Biological Control Laboratory – EPN production facility at RAB Rubona Station in Huye District.

Looking forward to a continuous good collaboration.

Yours sincerely,

Patrick KARANGWA (PhD)
Director General

Cc:
- Deputy Director General/Agriculture Research & Technology Transfer, RAB

P.O.BOX: 5016 KIGALI *Website:www.rab.gov.rw*