UNIVERSITY OF NAIROBI

CHARACTERIZATION AND EVALUATION OF ENTOMOPATHOGENIC NEMATODES FOR THE MANAGEMENT OF TOMATO LEAFMINER

(*Tuta absoluta* Meyrick)

BY

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DECLARATION

I declare that this thesis is my original work and has not been submitted elsewhere for examination or award of a degree. Where other peoples work has been used, this has been properly acknowledged and referenced in according with the University of Nairobi's requirements

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DEDICATION

To my parents, the late dad Ngugi Mirara and Wambui Ngugi for supporting girl child education, my husband, Peter Ndegwa for educating a wife, daughter Ruth and son Francis for your prayers and moral support throughout my postgraduate journey. God bless you all.

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ABBREVIATIONS AND ACRONYMS

ABD	-	Anal Body Diameter
ANOVA	-	Analysis of Variance
Bt	-	*Bacillus thuringiensis*
CABI	-	Commonwealth Agricultural Bureaux International
CAN	-	Calcium Ammonium Nitrate
CFIA	-	Canadian Food Inspection Agency
DAP	-	Double Ammonium Phosphate
DBM	-	Diamond Back Moth
DNA	-	Deoxyribonucleic acid
EB	-	Elution Buffer
ES	-	Pharynx Length
EP	-	Excretory Pore
EDTA	-	Ethylenediamine tetraacetic acid
EPNs	-	Entomopathogenic Nematodes
FAO	-	Food and Agriculture Organisation
G	-	Grams
GDP	-	Gross Domestic Product
GL	-	Gubernaculum Length
GOK	-	Government of Kenya
H	-	Hour
HCD	-	Horticultural Crops Directorate
ICIPE	-	International Centre of Insect Physiology and Ecology
IITA	-	International Institute of Tropical Agriculture
IJs	-	Infective Juveniles

IPM	-	Integrated Pest Management
IRAC	-	Insecticide Resisiatance Action Committee
ITS	-	Internal Transcribed Spacer
LB	-	Luria Bertani broth
KARI	-	Kenya Agricultural Research Institute
KALRO	-	Kenya Agricultural and Livestock Research Organisation
KEPHIS	-	Kenya Plant Health Inspectorate Services
MBD	-	Maximum Body Diameter
Min	-	Minutes
Ml	-	Milliliter
Mt	-	Metric tonnes
mM	-	micro Molecules
Mwt	-	Molecular weight
NA	-	Nutrient Agar
NBTA	-	Nutrient Agar Bromothymol blue Triphenyltetrazolium
PCPB	-	Pest Control Products Board
PCR	-	Polymerase Chain Reaction
PAGE	-	Polyacrylamide Gel Electrophoresis
pH	-	Potential of Hydrogen
PPSD	-	Plant Protection Service Division
PSC	-	Parliamentary Service Commission
rDNA	-	ribosomal DeoxyriboNucleic Acid
rRNA	-	ribosomal RiboxyNucleic Acid
Sec	-	Seconds
SL	-	Spicule Length

TAE	-	Tris Acetate EDTA
TAF	-	Trialamine Formaldehyde
TBL	-	Total Body Length
TEMED	-	Tetramethylethylenediamine
TL	-	Tail Length
TTC	-	Triphenyltetrazolium Chloride
UoN	-	University of Nairobi
UV	-	Ultra Violet
USDA	-	United State Department of Agriculture
USAID	-	United State Agency for International Development

ABSTRACT

Tomato (*Solanum lycopersicum* L.) is one of most known and grown vegetable globally. However, its production is constrained mainly by insect pests among them Tomato leafminer (*Tuta absoluta* Meyrick.) that causes yield losses of upto 100%. The objective of the study was to characterize and evaluate indigenous entomopathogenic nematodes (EPNs) isolates for the management of *T. absoluta* on tomato. Five indigenous EPN isolates (TK1, S86, 97, 75 and R52) at different concentrations (100, 150, 200, and 250) of infective juveniles (IJs)/ ml) were evaluated for infectivity on 2nd and 3rd larval stages of *T. absoluta* under laboratory conditions. Larval mortality (infectivity) was recorded at 24th h interval up to 120 h. The most infective isolate was subjected to morphological and molecular identification and associated symbiotic bacteria isolated and identified. The bacteria larvicidal effect on *T. absoluta* was evaluated and bacterial crude protein extracted and analysed through SDS-PAGE. All the tested EPN isolates infected and reproduced within *T. absoluta*. The EPN isolate TK1 was the most infective causing mortality100%; 92±5%), on 2nd and 3rd larval instars, respectively, at a concentration of 100 IJs/ml in the 48th h. The EPN isolate TK1 infective juveniles body was 834.54±87.36 µm (658.60-986.89 µm), hyaline 20.47±3.33 µm (13.45-24.95 µm) and tail 53.22±8.35 µm (40.30-71.51) µm in length. The 1st generation males were J shaped but strongly curved posteriorly almost spiral; spicule 82.05±13.94 µm (57.86-128.30 µm) and gubernaculum 45.28±5.89µm (34.48-54.87 µm) in length. Sequence analysis of ITS region of rDNA of EPN isolate TK1 revealed similarity (83% to 92%) with best BLASTNn hits with closest relative being *Steinernema* spp. (AY230186.1). The low similarity index result indicates EPN isolate TK1 could be a new species. Sequence analysis of 16S of rDNA symbiotic bacteria TK1, revealed similarity of 97 to 98.93% with best BLASTNn hits with *Xenorhabdus* sp. My8NJ (AB507811.1 being closest relative (98.93%). Mortality of 68% to 100% at 24 h at different concentrations against *T. absoluta* was achieved from bacteria isolate TK1 outside the host. The study concludes that EPN isolate TK1 is a *Steinernema* sp. while its symbiont bacteria TK1 is a *Xenorhabdus* sp. and both are pathogenic to *T. absoluta* larvae. From this study, the EPN isolate TK1, its symbiotic bacteria TK1 and bacterial crude protein are recommended for use in the management of *T. absoluta*. Further research is recommended to determine insecticidal compounds in the bacterial crude protein.

Key Words: Entomopathogenic nematodes; Infectivity; *Tuta absoluta*, *Steinernema* sp.; *Xenorhabdus* sp.; larvicidal activity

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Tomato production and constraints

Tomato (*Solanum lycopersicum* L.) belongs to the family Solanaceae. It is one of the world's most commonly and extensively grown edible fruit vegetable. In 2013, world tomato production was 163.4 million tonnes (Asgedom *et al.*, 2011). Kenya is ranked 6th in tomato production in Africa with a total production of 397,007 tonnes (FAO, 2012). Tomato is the second leading vegetable in production and value after potato which is mainly grown by small scale farmers in the country (Sigei *et al.*, 2014). In 2014, tomatoes were grown on 24,074 ha with a total production of 400,204 Mt worth Ksh 11.8 Billion (HCD, 2014). The vegetable grows in a wide range of conditions as a field crop or under controlled environment (green house). Tomato is an important food, rich in Vitamins A and C and lycopene, generation of revenue, foreign exchange earner and source of employment (Waiganjo *et al.*, 2013; Sigei *et al.*, 2014).

Despite the economic benefits of tomato especially in mitigating poverty, the vegetable is faced with abiotic and biotic constraints. Biotic challenges adversely affecting production and marketing of quality tomatoes include arthropod pests, diseases and physiological disorders (Waiganjo *et al.*, 2013; Mueke, 2014; Sigei *et al.*, 2014). Insect pests are estimated to contribute 34% of tomato biotic production constraints in Kenya (Ochilo *et al.*, 2019). Major tomato pests are African bollworm (*Helicoverpa amigera*), Spider mites (*Tetranychus* sp.), thrips (*Frankliniella occidentalis*), whiteflies (*Bemisia tabaci*) and the serpentine leafminer (*Liriomyza trifolii*) (Asgedom *et al.*, 2011; Waiganjo *et al.*, 2013; Mueke, 2014; Ochilo *et al.*, 2019). However, in early 2014 the South American leafminer (*Tuta absoluta*) was reported in Kenya (Daily Nation, 2014; FAO, 2014). The *T. absoluta* is a serious pest that attacks tomato from nursery to maturity causing severe yield losses reaching 100% (Ndalo *et al.*, 2015;

Shiberu and Getu, 2017; CABI, 2019). Currently, the *T. absoluta* is mainly controlled using insecticides such as Coragen (Chlorantraniliprole), Belt (Flubendiamide), Tracer (Spinosad) and Radiant (Spinetoram) (Hanafy and El-Sayed, 2013; PCPB, 2018). The pest is already showing resistance to many chemical insecticides but some biological and cultural strategies have been evaluated (Haddi *et al.*, 2012; Hanafy and El-Sayed, 2013). An integrated pest management (IPM) strategy that deploys all potential management options / approaches is required to enhance the control of *T. absoluta* (Illakwahhi and Srivastara, 2017).

1.2 Statement of the problem

Kenya's Vision 2030 stipulates agriculture as one of the economic pillars contributing 24% of Gross Domestic Product GDP). Agricultural productivity and competitiveness is constrained by low adoption of appropriate technologies among them pest management (GoK, 2010). Tomato is one of the world's most commonly and extensively grown edible fruit vegetable. In Kenya, tomato is the second leading vegetable in production and value after potato which is mainly grown by small scale farmers (Sigei *et al.*, 2014). Its production is costrained by physiological disorders, diseases and arthropod pests (Waiganjo *et al.*, 2013; Mueke, 2014; Sigei *et al.*, 2014). Among the arthropod pests of tomato is the Tomato leafminer*Tuta absoluta*. This is a migratory world wide pest, reported in 41 African countries including Kenya (CABI, 2019).

The pest damage occurs throughout the growing cycle of tomato (seedlings to maturity) (CFIA, 2016: Tumuhaise *et al.*, 2016). If not controlled, *T. absoluta* causes yield losses of up to 100% (Daily Nation, 2014; Ndalo *et al.*, 2015; Shiberu and Getu, 2017; CABI, 2019). Majority of tomato growers in the country use chemical insecticides to manage *T. absoluta*. Growers increase spray frequency to mitigate pest infestation which leads to chemical residues, threatening human health and environmental safety (Mueke, 2014). Resistance to commonly used pesticides on *T. absoluta* has been documented (Haddi *et al.*, 2012; Bala *et*

al., 2019; Guedes *et al.*, 2019; Ochola *et al.*, 2019). Use of pesticides costly to small scale growers. This calls for alternative *T. absoluta* effective management strategy, environmentally safe, and with reasonable cost benefits-especially for tomato growers. (Waiganjo *et al.*, 2013; Mueke, 2014).

1.3 Justification of the study

Kenya is ranked 6th in tomato production in Africa with a total production of 397,007 tonnes (FAO, 2012). As a major horticultural crop, its source food rich in Vitamins A and C and lycopene. In addition, the crop is important in revenue generation, foreign exchange earner and source of employment (Asgedom *et al.*, 2011; Waiganjo *et al.*, 2013; Sigei *et al.*, 2014). In the recent past, tomato farmers have been challenged by an invasive insect pest, *Tuta absoluta*, mainly managed by chemical pesticides. Chemical pesticides are costly and pose environmental and food safety concerns. Their use on *T. absoluta* is also limited due to the pests' nature of damage and its ability to develop insecticide resistant strains (Haddi *et al.*, 2012; Bala *et al.*, 2019).

There is thus need for integrated pest management (IPM) strategies in the management of *T. absoluta*. This include biological pest control agents among them entomopathogenic nematodes (EPNs). The EPNs have high virulence, safe to other organisms and efficacious in favourable habitats. The EPNs also are environmentally friendly, applied with conventional equipment and are applied together with most pesticides (Divya and Sankar, 2009; Aldamario *et al.*, 2010; Lacey *et al.*, 2015). In Kenya, EPNs have been commercially produced and used in the control of Sciarid flies, Thrips, Cutworms and Leafminers on ornamentals (PCPB, 2018). Research is still on going for new more virulent strains of EPNs, particularly indigenous species as they are considered to be well suited to local habitats (Kalia *et al.*, 2014; Nikdel and Niknam, 2015). According to Garcia-del-Pino *et al.*, (2013), 78.6-100% larval and 10% pupal mortality was achieved when EPN *Steinernema feltiae* was

evaluated against *T. absoluta* in laboratory and field trials. The objective of this study was therefore to characterize and evaluate indigenous EPN isolates for the management of Tomato leafminer (*Tuta absoluta*).

1.4 Research hypothesis

i. Entomopathogenic nematode (EPN) isolates 75, R52, 97, S86 and TK1 are infective on *Tuta absoluta* larvae in the laboratory conditions
ii. The most infective EPN among the study isolate can be characterized using morphometrical, morphological features and molecular methods
iii. The most infective EPN isolate harbours symbiotic bacteria
iv. The identified EPN symbiotic bacteria isolate has larvicidal effect on *T. absoluta* and has insecticidal crude protein

1.5 Objectives

1.5.1 Broad objective

To characterize and evaluate indigenous entomopathogenic nematode (EPNs) isolates for the management of *Tuta absoluta* on tomato for increased productivity

1.5.2 Specific objectives

i. To determine infectivity of five indigenous EPN isolates (75, R52, 97, S86 and TK1) on *T. absoluta* larvae in the laboratory
ii. To characterize the most infective EPN isolate using morphometrical, morphological features and molecular methods
iii. To isolate and identify symbiotic bacteria from the most pathogenic EPN isolate through biochemical and molecular methods
iv. To evaluate bacteria larvicidal effect of symbiotic bacteria isolate against *T. absoluta* in the laboratory and isolation of its crude protein

CHAPTER TWO

LITERATURE REVIEW

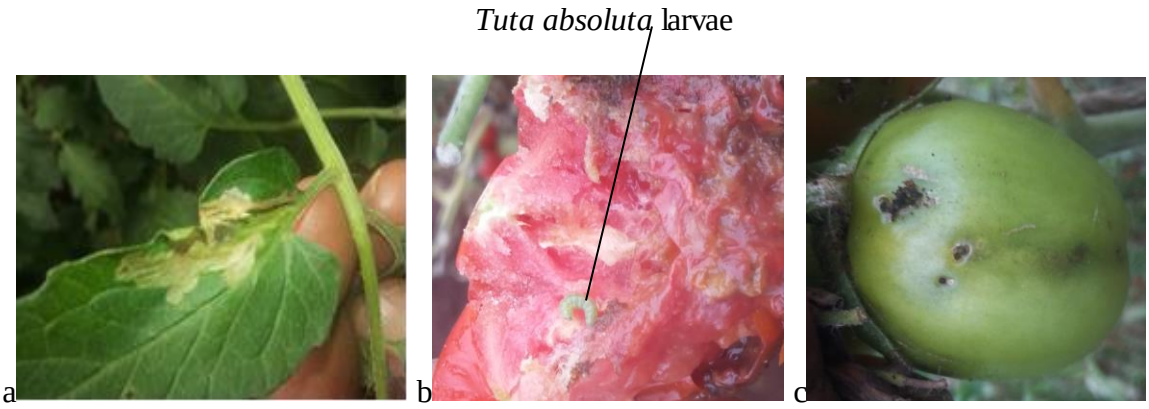
2.1 The biology and behavior of *Tuta absoluta*

The Tomato leafminer (*Tuta absoluta*) is a lepidopteran in the family Gelechiidae. It is a major pest of tomato that also infests a number of Solanaceae plants, and the common bean (Tumuhaise *et al.*, 2016; Mutamiswa *et al.*, 2017). The pest has crossed borders becoming a major tomato pest in many countries significantly devastating tomato production (Kaoud, 2014; CABI, 2019). *Tuta absoluta* is reported to have originated from South America (IRAC, 2011; Chidege, 2016; Kichaoui, 2016). The pest has high reproduction ability comprising of about 10-12 complete generations per year completing growth cycle in 30-35 days. The eggs are small cylindrical, creamy white to yellow, 0.35 mm long and hatch in 4 - 6 days after they are laid but darken just before hatching (Godfrey, 2016). Oviposition is on host plant foliage and lasts more than 20 days with females laying about 250-300 eggs singly or in small batches in her life (Desneux *et al.*, 2011; IRAC, 2011; USDA, 2011; Kaoud, 2014; Chidege, 2016).

The body of *T. absoluta* larvae is cream in appearance with a unique dark head (Retta and Berhe, 2015; Tumuhaise *et al.*, 2016). The moth of *T. absoluta* has a bead like antennae, a unique structure, scales that are silverfish-grey and anterior wing with characteristic black spots (USDA, 2011). The larvae changes colour from cream, greenish to light pink in the subsequent instars. The pest growth cycle is comprised of four larval stages which are very damaging and are completed in 12-15 days. The pest pupates mainly in the soil but also on leaf surface/folded /dry leaves and within tunnels (Chidege, 2016). The moths are 5-7 mm long with 8-10 mm wing span, and are mostly active at night (USDA, 2011; Retta and Berhe, 2015).

2.2 Damaging effect of *Tuta absoluta*

The pest can infest tomato from seedling to maturity affecting the overall crop quality and quantity. The *Tuta absoluta* larvae is the most damaging stage. The 1st and 2nd intars larvae feed on leaves by mining in the leaf mesophyll causing tunnels (Chidege, 2016; Kichaoui, 2016; Alam *et al.*, 2018). This leads to reduced leaf photosynthetic surface area translating to drying and premature death of the tomato plant (Kaoud, 2014). The 3rd and 4th instars feed by boring into tomato fruits, apical buds and stalks interfering with normal plant growth and productivity. Severe infestation leads to complete removal of leaf tissue leaving a skeleton of a dirtied excreta leaf (IRAC, 2011; USDA, 2011). Larvae may also form web like silken shelter on leaves tying them together further reducing photosynthetic surface area. Infested mature fruits are of low quality as they rot fast and fetch low prices in the market. Infested immature fruits have poor development and drop off prematurely. The injured plant parts are entry points for secondary pathogens hence further damage (IRAC, 2011) (Fig 2.1).



(Photo: Ngugi, C.N, 2019)

Figure 2.1 Damage of *Tuta absoluta* on tomato (a) leaf, (b) ripe and (c) immature fruit

2.3 History and distribution of *Tuta absoluta* in Kenya

Tuta absoluta was first described in 1997 as a native pest of South American mainly Peru, Bolivia and Chile (CABI, 2017). The pest was first reported in Spain (Europe) in 2006 and later in North America and other European Countries in 2010. According to Koskei, (2014),

the pest was reported in Africa in Morocco in 2007, Tunisia, Canar islands and Algeria in 2008; Senegal, Niger, Sudan and Ethiopia in 2012. In Tanzania and Uganda, the pest was reported in 2016 (Chidege, 2016; Tumuhaise *et al.*, 2016). In Kenya, existence of *T. absoluta* was reported through pest surveillance survey carried out in 2014 (FAO, 2014). This called for formation of a taskforce constituting of Kenya Plant Health (KEPHIS), Kenya Agricultural Research Institute (KARI), University of Nairobi (UoN) and Plant Protection Service Division (PPSD). The aim was to initiate a rapid survey in the upper regions of Eastern Kenya to establish the status of *T. absoluta* in the Country. The survey was conducted in tomato growing areas of Kirinyaga County (Mwea West and Kirinyaga West Sub-Counties), Meru County (Imenti South, Meru Central and Buuri Sub-Counties) and Isiolo County (Garbatulla and Isiolo Sub-Counties). According to Karanja, (2014), *T. absoluta* specific pheromone traps (Tutrack) and damage symptoms (on older leaves, coalesced mines forming large extended necrotic lesions) were used to establish presence of the pest. The survey results confirmed presence of *T. absoluta* in Kenya. Pest awareness programs through the media, workshops and farmer schools have been organized to sensitize farmers on the pest. The pest has in the recent past been reported in other parts of the country including Nairobi, Nakuru, Kakamega, Lamu, Loitokitok and Marsabit (CABI, 2015).

2.4 Integrated pest management options for *Tuta absoluta*

2.4.1 Chemical control

Management of *T. absoluta* is mainly by conventional insecticides (Bala *et al.*, 2019; Guedes *et al.*, 2019). This has led to the enormous use of chemicals by tomato farmers (Desneux *et al.*, 2011; Abbes and Chermiti, 2012). Insecticides mainly used against *T. absoluta* include Spinosin, Indoxacarb, Abamectin, Emamectin benzoate and Cyromazin (Berxolli and Shahini, 2018). In Kenya, *T. absoluta* control strategies for include use of insect traps (TUTRACK) a sex pheromone trap for early pest detection. Other registered insecticides

include BELT (Flubendiamide), EVISECTS (Thiocyclam) and TIHAN (Spirotetramat) (PCPB, 2018). Due to the damaging nature and reproduction ability of this pest, insecticides offer low to moderate effect on the insect with insecticide resistance being reported (Haddi *et al.*, 2012; Bala *et al.*, 2019).

2.4.2 Cultural control

Cultural measures include proper ploughing during land preparation, manuring, irrigation, crop rotation, solarisation, and the removal and destruction/disposal of infested (rogueing) marerials. Removal and destruction of host plants mainly the Solanaceae family is also advised. Regular pest population monitoring through scouting is also recommended. After crop harvest *T. absoluta* infested greenhouses should remain closed to prevent adults from escaping (Abbes *et al.*, 2012; Retta and Berhe, 2015; CABI, 2019). Use of insect exclusion nets, closure of green house doors and limited human traffic are also recommended (Giri and Sah, 2017; CABI, 2019)**.**

2.4.3 Semiochemicals

Pheromone traps are essential for monitoring *T. absoluta* population and management in open and indoor crops (Cocco *et al.*, 2013). These traps work in combination with other pest control measures, pheromone -baited traps, mating disruption or lure and kill techniques.

2.4.4 Biological control

This is use of living organisms in pest management which include predators (mirid bugs *Nesidiocoris tenuis* and *Macrolophus pygmaeus*). Efficacy of *Bacillus thuringiensis* (Bt) against the *T. absoluta* first-instar larvae has been reported (Alsaed *et al.*, 2017; Hosseinzadeh *et al.*, 2019).

Entomopathogenic fungi (*Metarhizium anisopliae* and *Beauveria bassiana*) are reported to attack *T. absoluta* with *M. anisopliae* causing 54% mortality of adults (Kichaoui, 2016; Alikhani *et al.*, 2019). Laboratory and field assessment on effect of entomopathogenic

nematode *Steinernema feltiae* on *T. absoluta* has been done with 78.6-100% larval and 10% pupal mortality being reported (Garcia-del-Pino *et al.*, 2013). Potential use of EPNs as biological agents of tomato pests including those that form galleries was reported by Garcia-del-Pino *et al.*, (2018).

2.4.4.1 Entomopathogenic nematodes

The entomopathogenic nematodes (EPNs), are useful biocontrol agents for pests and other harmful microorganisms (Valadas *et al.*, 2014; Shapiro-Ilan *et al.*, 2015). They are known since the 17th Century but it was not until 1930s that they were considered for use in pest control. They are a nematode-bacterium complex with *Steinernema* and *Heterorhabditis* species being the most important biological pest control agents (Cruz-Martinez *et al.*, 2017; Kagimu *et al.*, 2017). The EPNs have been used as biological control on indoor and field crops insect pests. They have a wide hosts range, high virulence, safety to other organisms and efficacious in favourable habitats. The wide host range, ease of multiplication / production and stability in formulation and mode of application has made EPNs become competitive with conventional insecticides (Divya and Sankar, 2009; Aldamario *et al.*, 2010; Lacey *et al.*, 2015). The EPNs also are environmentally friendly, applied with conventional equipment and are applied together with most pesticides. These nematodes reproduce in the insect host body, this provides the next generation of infective juveniles (IJs) that infest other insects hence a naturally self-sustaining process (Kagimu *et al.*, 2017).

The EPNs infect host insect through natural body openings or by direct penetration through the cuticle/skin especially for *Heterorhabditis* species. Once in the host body the IJs release the symbiotic bacteria, which multiply in the hemolymph and the insect dies within a few days (24-72 h) due to the bacteria and toxins produced by the nematodes (Hinchliffe *et al.*, 2010; Kalia *et al.*, 2014; Berbercheck, 2015; Kagimu *et al.*, 2017). Nematode growth and reproduction depend upon host immune response and conditions established by the

bacterium. The bacterium produces anti-immune proteins to assist the nematode in overcoming host defenses, and anti-microbials that suppress growth of secondary invaders (Kagimu *et al.*, 2017). The mutualistic bacteria and their metabolic by-products nourish EPNs in the feeding third juvenile stage (Shapiro-Ilan *et al.*, 2015). Use of EPNs in pest management has been mainly on soil dwelling insects but over time research shows their potential use against foliar feeding pests (Lacey *et al.*, 2015; Nikdel and Niknam, 2015; Platt, 2017). According to Akyazi *et al.*, (2012), over 60 species of *Steinernema* and 16 species of *Heterorhabditis* have been identified. Search for new strains of indigenous EPNs continues as they are better adapted to local conditions.

The EPNs as biocontrol agents have been used to suppress Rice leaf folder (*Cnaphlocrosis medinalis* Goen.), and Peach tree borer (*Synathedon exitiosa*) (Sankar *et al.*, 2009; Shapiro-Ilan *et al.*, 2015). Pest suppression of at least 80% has been reported using EPNs under field conditions. According to Noujeim *et al.*, (2015), the Pea leafminer pupae (*Liriomyza huidobrensis*) was susceptible to EPN *Heterorhabditis indica* in laboratory conditions.

2.4.4.2 Entomopathogenic nematodes symbiotic bacteria *Photorhabdus* and *Xenorhabdus*

Entomopathogenic nematodes *Steinernema* and *Heterorhabditis* sp. live mutualistically with bacteria in the genus *Xenorhabdus* and *Photorhabdus*, respectively (Gulcu *et al.*, 2012). These bacteria are source of nourishment to EPNs and have a generally similar life cycles (Aly and Mona, 2009; Sternberg and Dillman, 2012; Salgado-Morales *et al.*, 2019). The EPNs symbiotic bacteria are members of family Enterobacteriaceae the purple bacteria gamma subdivision and which are motile (Hinchliffe *et al.*, 2010; Salgado-Morales *et al.*, 2019). They colonize the intestines (receptacle or extended region) of the infective juveniles (IJs) stage of EPNs.

The *Photorhabdus* bacteria lights under culture stationary phase in the infected insect host/cadaver (Singh *et al.*, 2012). According to Hinchliffe *et al.* (2010) and Rodou *et al.*, (2010), the genus *Photorhabdus* is comprises three species, *P. luminescens*, *P. temperata* and *P. asymbiotica*. Further classification has placed *P. luminescens* and *P. temperata* species into subspecies based on DNA-DNA relatedness and 16S rDNA branching (Hinchliffe *et al.*, 2010; Peat *et al.*, 2010). The *Xenorhabdus* spp. bacteria are catalase negative, gram negative and rod shaped. The genus consists of five species: *X. beddingii, X. bovienii, X. japonicus, X. nematophilus* and *X. poinarii*. Based on 16S rDNA gene sequencing molecular and morphological characterization bacteria species from other isolates of *Steinernematidae* nematodes have been identified (Sangeetha *et al.*, 2016). The number of EPN species is likely to increase as nematodes continue to be collected from around the world and their symbiotic bacteria isolated and identified.

2.4.4.3 Bacterial protein toxin

Comparative genomic studies on different EPNs symbiotic bacteria, *Xenorhabdus* and *Photorhabdus* species has highlighted presence of numerous genes implied in their insecticidal action (Ruiu *et al.*, 2013; Stock *et al.*, 2017). The EPNs, associated bacteria and toxins when injected into the insect hemocoel are well known for their ability to kill insects (Quiroz-Cotaneda *et al.*, 2015). These properties contribute to the EPNs pathogenicity; however, for utilization it is important that a toxin is active when fed to insects. Protein toxins of different molecular weight from EPN bacteria *Photorhabdus and Xenorhabdus* species with oral insecticidal properties or by injection against Greater wax moth (*Galleria mellonella*) have been isolated (Jang *et al.*, 2011; Shi *et al.*, 2012; Castgnola and Stock, 2014).

2.4.4.4 Entomopathogenic nematodes in Kenya

Information on indigenous EPNs in Africa is still scanty. Currently, documented studies are only available from Kenya, Egypt, Ethiopia, Nigeria, Cameroon and South Africa (Waturu, 1998; IITA, 2008; Mwaniki, 2009; Akyazi *et al*., 2012). In Kenya, information on EPNs was first documented following a survey in Central highlands and Coastal counties where 154 nematodes were isolated. Only a few of the isolates including *Steinernema karii* have been identified (Waturu *et al*., 1997; Waturu, 1998; Mwaniki, 2009). More nematode isolates (12 isolates) have been collected from subsequent survey in the Rift Valley (Mwaniki, 2009). According to Kawaka *et al*., (2011), EPNs diversity and distribution is influenced by land use. Different EPNs dominate a particular habitat and locality and thus identification of indigenous species is important. The *Steinernema* species has been found to be dominant in Embu and Taita Counties (Kawaka *et al*., 2011).

Research in Kenya, has confirmed susceptibility of the Sweet potato weevil (*Cylas puncticollis*), larvae on sweet potatoes vines and tubers to both *Heterorhabditis indica* and *Steinernema karii* (Waturu, 1998; Nderitu *et al*., 2009). The *H. indica* and *S. karii* are pathogenic against Banana weevil (*Cosmopolites sordidus* Germar), in the field and *S. yirgalemense* in the laboratory (Waturu *et al*., 2006; Ndiritu *et al*., 2016). The EPNs *S. weiseri*, *H. indica,* and *S. karii* have been found to be lethal on Diamond back moth (*Plutella xylostella*) in kales under laboratory and field conditions (Nyasani *et al*., 2008). Susceptibility of Flower thrips on French bean to EPNs *Steinernema karii* and *Heterorhabditis bacteriophora* has been documented (Ng'ang'a *et al*., 2011). Susceptibility of legume podborer (*Maruca vitrata*) to different concentrations of EPNs *H. indica* and *S. karii* in the laboratory and semi field trials has been reported by Ngugi, (2012).

2.4.4.5 Unidentified entomopathogenic nematode isolates from Kenya

Commercial applications of EPNs so far has been reported in the management of pests in flower industry but no report on EPN bacterial use in Kenya. Efficacy evaluation and identification work by scientists from different institutions has continued over years but a number of the isolates remain unknown together with their bacterial larvicidal effects on insect pests. The EPN isolates 75, 97, S86, R52 and TK1, are currently (2020), maintained at KALRO Kandara but the isolates identity, pathogenicity, their symbiotic bacteria, bacteria effect on *T. absoluta* is unknown thus reason for this study.

CHAPTER THREE

GENERAL MATERIALS AND METHODS

3.1 Study Site

The study was conducted at the Kenya Agricultural Livestock Research Organisation (KALRO), Kandara, International Centre for Insect Physiology and Ecology (ICIPE) and University of Nairobi (UON), Kenya.

3.2 Establishment and maintenance of tomato plants

Two tomato varieties Milele F1 and Rio Grande were established for the purpose of providing feeding materials for *Tuta absoluta*. The Milele F1 is semi determinate hybrid variety, maturing in 90 days and with vigorous growth. It is tolerant to bacterial and Fusarium wilt and Tomato Leaf Curl Virus (Fresco seed, 2016). The Rio grande variety is determinate with bush type growth habit and it matures in 75-85 days. Planting was done on 27th March, 2017 in screen house size 30 m x 33 m. Planting holes were filled with a mixture of top soil, rotten farmyard manure and 10 g of Double Ammonium Phosphate (DAP) fertilizer. The plants were irrigated through drip system daily in the first week and on alternate days thereafter. Top dressing with 5 g of Calcium Ammoniun Nitrate (CAN), gapping and trellising were carried out two weeks after transplanting.

3.3 Tomato leafminer (*Tuta absoluta*) culture

Tuta absoluta life stages (larvae, pupa and adults), were collected from infested tomato farms in Murang'a, Embu, Kirinyaga and Laikipia Counties to establish insect culture at KALRO Kandara. The life stages were reared on tomato established in a screen house. Fresh *T. absoluta* instars were regularly collected and introduced to maintain insect culture hybrid vigour.

3.4 Rearing of Greater wax moth (*Galleria mellonella*)

The greater wax moth (*Galleria mellonella*) larvae were used to multiply and maintain EPN cultures in the laboratory. Different life stages (eggs, pupae and larvae) of *G. mellonella* were used to establish a laboratory insect culture. The life stages were obtained locally from bee keeping farmers honey combs and from an existing laboratory culture at KALRO-Kandara. The insect was reared on artificial diet comprising of bee wax 45 g, brewer's yeast 95 g, maize flour 307 g and pure honey 225 g (Waturu, 1998). Honey wax was pre-melted in a pot and honey added while stirring vigorously using a wooden spoon. Maize flour and yeast were added to the mixture of honey and bee wax and mixed thoroughly. The mixture was transferred to a ventilated plastic container and allowed to cool before introducing life stages of greater wax moth. The boxes were incubated at 25±2°C for about 4-5 weeks when eggs hatched to start the culture. For long storage, *G. mellonella* larvae were rinsed in water heated for 20 Sec at 60°C and in cold water for 10 sec.

3.5 Entomopathogenic nematode isolate cultures

The entomopathogenic nematodes (EPN) culture was established and multiplied *in vivo* using *Galleria mellonella* larvae reared as described in section 3.4. The *G. mellonella* is preferred due to its availability, size, ease in laboratory rearing on diet and high susceptibility and yield to a range of EPNs (Divya and Sankar, 2009). An unidentified indigenous nematode isolates coded 75, 97, R52, S86 and TK1 were randomly selected from an existing culture. The nematodes had been earlier isolated from soils in Central highland Counties and Kwale County of coastal region, Kenya. Larvae of the greater wax moth were used as baits for the nematodes (Shapiro-Ilan *et al.*, 2012; Lacey *et al.*, 2015). White trap method was used to trap EPNs White, 1927). Suspensions of EPN isolates infective juveniles (IJs) were drawn from a stock suspension stored at 20°C and conditioned to room temperature (23-25 °C) for at least 1h. Viability of the EPN isolates was assessed by placing part of the suspension into a Petri

dish and observing under compound microscope. A volume of the suspension was diluted with appropriate amount of distilled water. The diluted nematode suspension was adjusted to 200 nematodes per ml that was dispensed into a 9 cm Petri dish. Ten pre–pupating larvae of *G. mellonella* were placed in each Petri dish which was later covered with a lid (Fig 3.1).

Photo: Ngugi, CN, 2019

Figure 3.1 *Galleria mellonella* larvae baited with five entomopathogenic nematode isolates

The Petri dishes were incubated at room temperature of 25±2 °C until the death of the *G. mellonella* larvae. The cadavers were later transferred into modified White traps assembled by placing an inverted plastic Petri dish in a 250 ml plastic container, white cotton cloth and distilled water. The white cloth was placed in such a way that one edge touched the distilled water in the plastic container. Infected *G. mellonella* cadavers were then arranged in a circular manner, close to each other on the modified White trap with the anterior/ head of the cadaver facing up and posterior/rear side towards the distilled water (Fig 3.2) for all the study isolates.

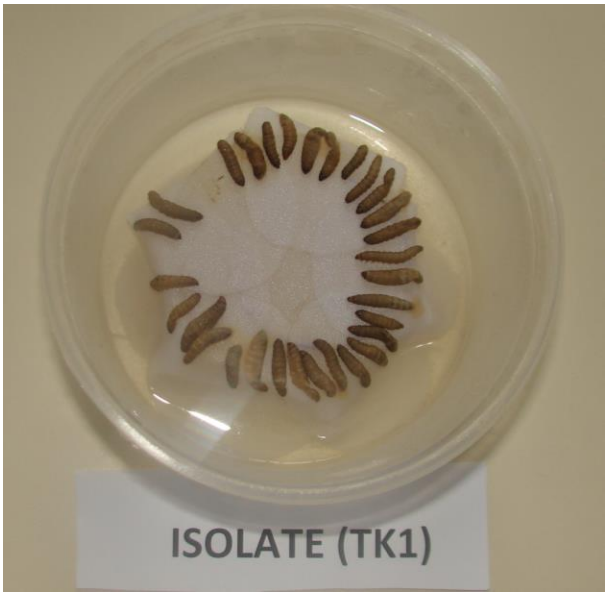


Photo: Ngugi, CN, 2019

Figure 3.2 Trapping of entomopathogenic nematode isolates from *Galleria mellonella* larvae

The infective juveniles (IJs) emerged from the cadavers in about 7-10 days later, moved through wet white cloth and were harvested from the distilled water. The IJs were cleaned three times in distilled water. Freshly harvested IJs at a concentration of about 100 ml were then stored in 1 cm deep distilled water in 500 ml plastic containers well labeled with isolate number/code and date of harvesting. The containers were stored on shelves in the dark at room temperature of 25±2 °C for short term use and 20 °C in an incubator for long storage.

3.5.1 Determination of nematode concentration

Nematode doses were prepared from a stock suspension stored at 20°C. A known volume of nematode suspension was transferred into a 250 ml beaker and shaken to uniformity. Five aliquots of 1 ml each of nematode suspension were drawn and counted in nematode counting dishes (Fig 3.3). Counting of nematodes in 1 ml was done three times and the mean number was determined. To increase the concentration or number of nematodes per ml, the suspension was left to settle for 1h and a given volume decanted. Where nematode concentration per ml was higher than the required, more distilled water was added to the stock suspension.

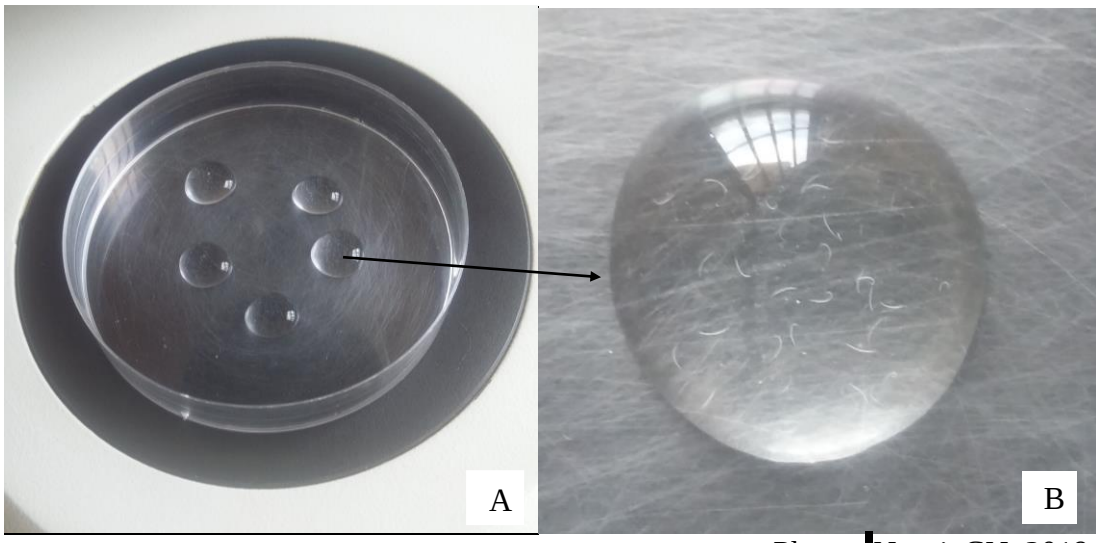


Photo: Ngugi, CN, 2019

Figure 3.3 Entomopathogenic nematodes dose determination (A) 1 ml of five drops (B) single drop of suspension (X20)

3.6 Media preparation

The media were prepared according to manufacturer instructions. Nutrient agar (NA) media 15 g (3.0 g beef extract, 5.0 g peptone and 15 g agar), was dissolved into 1000 ml of distilled water. MacConkey agar, 50 g of the powder (17.0 g pancreatic digest gelatin, 2.0 g peptones, 10 g lactose monohydrate, 5.0 g bile salts, 5.0 g Sodium chloride, 0.075 g neutral red and 12.0 g agar) was dissolved in 1000 ml of distilled water. For Nutrient Bromothymol blue Triphenyl-tetrazolium chloride agar (NBTA), 8.0 g nutrient agar, 25 mg bromothymol blue and 40 mg of 2,3,5-triphenyltetrazolium) were dissolved in 1 litre of distilled water. Nutrient broth (NB), 13 g (Peptone 5.0 g, Yeast extract 3.0 g of beef extract 1.0 g and Sodium chloride 5.0 g) were added in a litre of distilled H_2O heated to dissolve for a min. The free flowing solution was dispensed after cooling to 50°C. About 24 g of Urease agar (urea 20.0 g, Sodium chloride 5.0 g, Monopotassium Phospahate 2.0 g, Phenol red 0.012 g and agar 15.0 g at pH 6.7±0.2) was dissolved in 950 ml of distilled water. This media was cooled to 50-55°C and 4-5 ml dispensed in sterile tubes and slanting the tubes during cooling until solidified. All the

media were autoclaved for 15-20 min at 121°C. Un-inoculated plates with media were incubated at 35-37°C for 48 h to test for media sterility.

3.7 DATA ANALYSIS

The data on mortality was subjected to Statistical analysis, Analysis of variance (ANOVA) using Genstat, 15th edition, Statistical software. The Means were separated using Fisher's protected least significant difference test at 5% significance level. Data was summarized in tables, graphs and also presented in photographs.

CHAPTER FOUR

LABORATORY SCREENING FOR INFECTIVITY OF FIVE ENTOMOPATHOGENIC NEMATODE ISOLATES ON *Tuta absoluta*

4.1 INTRODUCTION

Tomato (*Solanum lycopersicum* L.) belongs to the family Solanaceae and is one of most known and grown vegetable globally. Its production is faced with many constraints including diseases and insect pests (Waiganjo *et al*., 2013; Wakil *et al*., 2018). In the recent past, devastating pest, the Tomato leafminer (*Tuta absoluta*) has gained importance mainly in the cultivated Solanaceae plants, tomato being the major host.

The pest is native to South America from where it has spread to Europe, Asia, Middle East, East and South Africa (Desneux *et al*., 2011; Retta and Berhe, 2015; Kichaoui *et al*., 2016; Mutamiswa *et al*., 2017; Visser *et al*., 2017). The pest can cause economic losses of up to 100% in out and indoor tomato production (Retta and Berhe, 2015; Mutamiswa *et al*., 2017). In Kenya, *T. absoluta* was first reported in 2013 in Isiolo, Embu, Meru, Garissa, Wajir and Marsabit Counties from where it has spread to all tomato growing regions in the country. The pest is believed to have entered the country from Ethiopia where it had been reported earlier (Daily Nation, 14.06.2014).

The current management of *T. absoluta* in most parts of the world is mainly by chemical with farmers using 8-25 sprays on tomato in a season (Gozel and Kasap, 2015; Retta and Berhe, 2015; Roditakis *et al*., 2015; PCPB, 2018). Use of synthetic pesticides poses health and environmental safety concerns. Also effectiveness of chemical control is limited due to *T. absoluta* nature of damage and its ability to develop insecticide resistant strains (Haddi *et al*., 2012; Roditakis *et al*., 2015; Bala *et al*., 2019). Other management and control measures of *T. absoluta* include cultural, physical and use of biological agents among them entomopathogenic nematodes (EPNs)

The EPNs are free living roundworms in the family Steinernematidae and Heterorhabditidae that parasitize and kill many insect species (Hoctor, 2013; Valadas *et al.*, 2014, Nikdel and Niknam, 2015, Garcia-del-Pino *et al.*, 2013). These nematodes were first reported in Kenya in 1997 after a survey in Coastal and Central region (Waturu, 1998). Use of EPNs in pest management has been mainly on soil dwelling insects but over time research shows their potential use against foliar feeding pests (Lacey *et al.*, 2015; Nikdel and Nikman, 2015; Platt, 2017).

Effectiveness of EPNs in pest management depends on pest lifecycle, environmental conditions during application, range of hosts, foraging ability and matching of species among others (Lacey and Georgis, 2012; Garcia-del-Pino *et al.*, 2013; Hoctor, 2013; Nikdel and Niknam, 2015). These nematodes infect hosts by entering the body through orifices or directly through the cuticle in case of *Heterorhabditis* spp. The EPNs then release symbiotic bacteria which reproduces inside the host's body, killing them within 24 to 72 h (Salvadori *et al.*, 2012; Shapiro *et al.*, 2012; Hoctor, 2013; Kalia *et al.*, 2014; Berbercheck, 2015). Mortality/death is only in pests susceptible to EPNs infectivity. Thus, correct host–nematode combination is vital in enhancing infectivity of nematodes in the pests. Nematode infectivity, is determined by the percentage number of infected pest larvae which is indicated by their mortality (Dobes *et al.*, 2012; Berbercheck, 2015). The rate of infectivity is also affected by EPNs dosage and time. Low dosage gives reduced host mortality while a high one may lead to infection failure. A dose of 25-200 infective juveniles (IJs) / larvae is adequate to evaluate EPNs infectivity against *Galleria mellonella* (Woodring and Kaya, 1988; Murad *et al.*, 2019). Indigenous strains of EPNs active against *T. absoluta* represent a reliable alternative to excess and inappropriate use of chemical insecticides for the management of this pest. Successful incorporation of EPNs in integrated pest management (IPM) is dictated by successful evaluation of novel species or strains for efficacy/infectivity against a target pest (Shapiro-

Ilan *et al.*, 2012). The objective of this study was therefore to determine the infectivity of five indigenous EPN isolates on *T. absoluta*, based on time, dosage and their reproduction potential (progeny recovery).

4.2 MATERIALS AND METHODS

4.2.1 Entomopathogenic nematodes and *Tuta absoluta* cultures

The entomopathogenic nematode (EPN) isolates for this study (S86, 75, 97, R52 and TK1) were obtained from a stock maintained at Kenya Agricultural and Livestock Research Organisation (KALRO), Kandara. The EPNs had been earlier isolated from soils in a survey in Central highland counties and Kwale County of coastal region, Kenya (Waturu, 1997). They were reared and multiplied on greater wax-moth (*Galleria mellonella*) in the laboratory at 24 ±2°C according to Woodring and Kaya, (1988). Suspensions of infective juveniles (IJs) of EPN isolates were drawn from a stock suspension stored at 20 °C and conditioned to room temperature for an hour. Survival of the EPN isolates was observed using a compound microscope. The EPNs suspension concentration was adjusted by dilution using distilled water. The diluted suspension of each of the EPN isolates was adjusted to 200 nematodes per ml which was added into a 9 cm Petri dish padded with a white cotton cloth. Ten pre–pupating larvae of *G. mellonella* were placed in each Petri dish. After five days of infection (baiting), IJs were recovered from *G. mellonella* using modified White trap method according to White, (1927). The recovered IJs were stored as suspension in 50 ml distilled water in 250 ml plastic at 20 ±2°C for later use. The *T. absoluta* were maintained on tomato crop established in a screen house at KALRO Kandara, from where the 2nd and 3rd larval instars were obtained.

4.2.2 Infectivity of entomopathogenic nematode isolates against *Tuta absoluta*

Insect mortality bioassay was carried out using five different EPN isolates: S86, 75, 97, R52 and TK1. Five larvae of *T. absoluta* were placed singly in a 9 cm petri dish lined with white

cotton cloth for each replicate per concentration of each isolate. The EPNs were introduced to second and third instars of *T. absoluta*, larvae. The experiment design was completely randomized design (CRD) with five treatments; control (distilled water without EPNs), 100, 150, 200 and 250 of IJs per ml of distilled water. In the control treatment only 1 ml of distilled water was used to wet the filter papers before placing the *T. absoluta* larvae. Each treatment was replicated five times with five larvae for each replicate (N=25). Dead *T. absoluta* larvae were randomly selected from each treatment and dissected under the microscope to confirm mortality due to EPNs. Data on mortality against time and concentration were recorded in each EPN isolate at 24 h interval for 5 days. Data on mortality against exposure time and concentration was subjected to Analysis of Variance (ANOVA) Statistical analysis using GenStat Software, 15th edition. The means were separated using Fisher's protected least significant difference test at 1% significance level.

4.3 RESULTS

4.3.1 Entomopathogenic nematode infected fresh *Tuta absoluta* cadavers

From this study, EPN isolates 75, 97, S86, R52 and TK1 caused death of *Tuta absoluta* larvae at different time of exposure and concentrations. The cadavers of larvae were obtained and emergence of infective juveniles from cadavers was also observed5th day after infection (Fig 4.1 A and B).

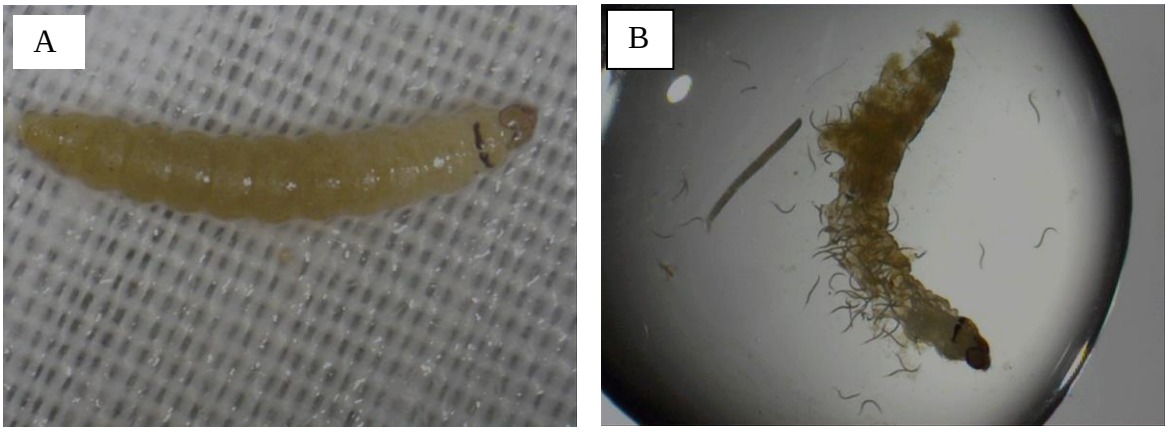


Photo: Ngugi, CN. 2019

Figure 4.1 (A) Fresh nematode infected *Tuta absoluta* cadaver and (B) Infective juveniles emerging from a *T. absoluta* cadaver (X40)

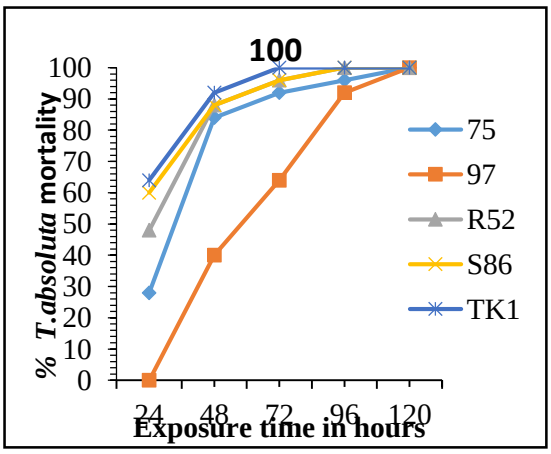
4.3.2 Effect of entomopathogenic nematode isolate concentrations and time on mortality of *Tuta absoluta* 2nd instar larvae

In the control experiment, there was no mortality observed over 120 h. The nematode suspension concentration of 100 IJs/ml distilled water, at exposure time of 24, 48 and 72 h, EPN isolate TK1 had the highest percent mean mortality of 60±8.9, 92±4.9 and 100±0.0% respectively. The EPN isolate 97 had the least means at exposure time of 24, 48, 72 and 96 h (20±0, 40±8.0, 60±13, 76±4.0 and 96±4.0%). Mortality of 100% was observed in isolate TK1 from 72nd h, and isolates S86 and R52 from 96th h (Fig 4.2A). There was no significant difference (P>0.001) between isolates in all the exposure time for concentration 100 (Appendix 1).

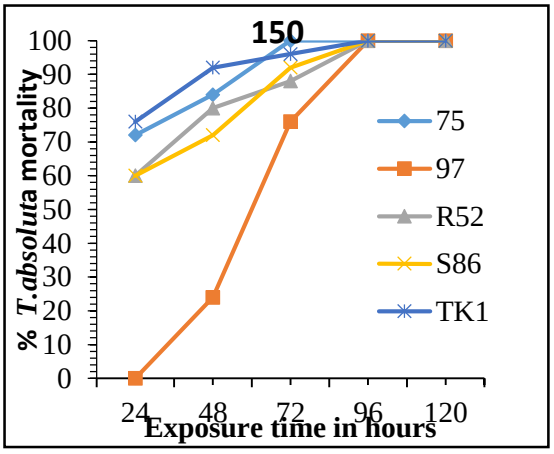
The EPN isolate TK1 recorded highest % mean (60±8.9 to 100%) at concentration 150 in all exposure time although a 100% mortality was reached at 72nd h. Isolate 97 caused least mortality (12±4.9, 36±4.0 and 68±12%) by 72nd h. All the isolates recorded >50% mortality by the 72nd h (Fig 4.2.B). There was significant difference (P<0.0001) between TK1 with all the other EPN isolate at 24 and 48 h (Appendix 2).

In EPN concentration 200, highest mean of 60±11.0% was observed in EPN isolate S86 in the 24th h but no significant difference (P>0.0001) was recorded from other isolates. Isolate 97 recorded lowest % mortality of 12±8, 44±8 and 68% at 24th, 48th and 72nd. There was significant difference (P<0.0001) between isolate S86 and 97 and later with the all isolates in the 24th h (Appendix 3). All isolates had >50% mortality in the 72nd h and 100% by the 96th h (Fig 4.2.C).

From 24 to 96 h of exposure, in concentration of 250, EPN 97, caused low mortality (4±4, 40±8.9 and 76±9.8), among the isolates. The isolate was significantly different (P<0.0001) from the rest of isolates at 24 and 48 h only. In the 72 h there was significant difference (P<0.0001) between EPN 97 and R52, S86 and TK1 (Appendix 4). More than >50% mortality was registered by the EPNs in the 72 h with R52 recording 100%. All isolates attained 100% larval mortality by the 120th h (Fig 4.2.D).



A



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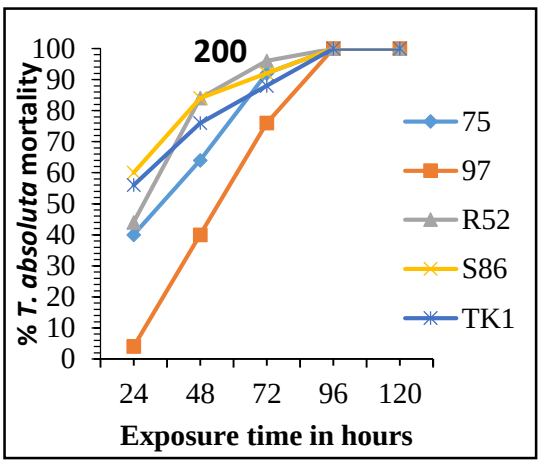

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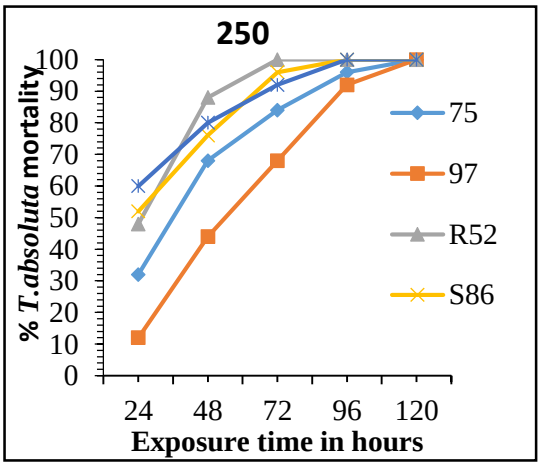


D

Figure 4.2 Infectivity of entomopathogenic nematode isolates at different nematode concentrations (A 100, B 150, C 200 and D 250) on 2nd larvae of *Tuta absoluta*

4.3.3 Effect of entomopathogenic nematode isolate concentrations and time on mortality of *Tuta absoluta* 3rd instar larvae

In the control experiment, there was no mortality observed over 120 h. At concentration 100 of IJs/ml distilled water, at the 24th h, TK1 had the highest larval mortality of 64±12%, followed by EPN isolate S86, R52, 75 and 97 with 60±0.0, 48±4.9% 28±10.2 and 0±0%, respectively. There was significant difference ($P<0.001$) between isolate 75, 97, and R52. There was no significant difference ($P>0.001$) between R52, S86 and TK1 at the 24th h (Table 4.1). In the 48 and 72 h of exposure, isolate TK1 had a mean of 92±5; 100±0.0% respectively with significant difference ($P<0.001$) between TK1 and 97 in both h. Mortality of >90 percent in all the isolates was recorded in 96 and 120 h (Table 4.1).

At concentration 150, on the 24 h, TK1 caused the highest larval mean mortality of 76±0.0% followed by isolate 75, S86, R52, and 97 (72±14.97, 60±10.95, 60 and 0±0 %, respectively). There was significant difference ($P<0.001$) between EPN 97 and all the others. In the 48th h TK1 also led with a mean mortality of 92±0.0% followed by isolate 75, R52, S86 and 97 with mean mortality of 84±7.5, 80±8.97, 72±4.9 and 24 ±7.5%, respectively. The EPN 97 was significantly different ($P<0.001$) from the other isolates. In the 72nd h, highest mean

(100±0%) was observed in EPN isolate 75; while isolates TK1, S86, R57 and 97 the order of means was 96%, 92±8, 88±8 and 76±14.7%. There was significant difference (P<0.001) between S86 and TK1. In the 96th and 120th h mortality was 100 percent in all the isolates (Table 4.2).

In concentration 200, isolates R52 at h 24, 48 and 72 gave highest mean larval mortality (64±12; 96±4%). The isolate was significantly different (P<0.001) from isolate 97 in the 24 and 72 h and TK1in the 48th h (Table 4.3). All the isolate recorded larval mean mortality of ≥80% by the 72nd h but 100% mortality in 96th and 120th h (Table 4.3).

Concentration 250 EPNs TK1, S86, R52, 75 and 97 had mortality of 64±4.0, 56±8.0, 52±10.19, 40±8.9 and 0 ±0 % in the 24th h (Table 4.4). Significant difference (P<0.001) was between TK1 and 97 and 75 in the same hour. In the 48th h, isolates 97 and 75 had the least means of 40±12.6 and 76±7.5% respectively. There was significant difference (P<0.001) between isolate 97 and the other EPN isolates, and isolate TK1 with 97, R52 and S86 in the 48 h. All the isolates recorded a mean of ≥60% in the 72nd h but 100% was observed in the 120th h (Table 4.4).

Table 4.1 Mortality of 3rd instar larvae of *Tuta absoluta* over time at 100 concentration of entomopathogenic nematode isolates (Mean ± SEM)

EPN Isolates	Exposure time (h)				
	24	48	72	96	120
75	28±10.2^{d}	84±7.5^{a}	92±4.9^{a}	96±4.0^{a}	100±0.0^{a}
97	0±0.0^{e}	40±14.1^{cd}	64±14.7^{b}	92±4.9^{a}	100±0.0^{a}
R52	48±4.9^{bc}	88±8.0^{a}	96±4.0^{a}	100±0.0^{a}	100±0.0^{a}
S86	60±0.0^{b}	88±8.0^{a}	96±4.0^{a}	100±0.0^{a}	100±0.0^{a}
TK1	64±11.7^{b}	92±4.9^{a}	100±0.0^{a}	100±0.0^{a}	100±0.0^{a}
P value	< 0.001	< 0.001	< 0.001	> 0.001	>0.001

Means within a column with the same letters are not significantly different

Table 4.2 Mortality of 3rd instar larvae of *Tuta absoluta* over time at 150 concentration of entomopathogenic nematode isolates (Mean ± SEM)

EPN isolates	Exposure time (h)				
	24	48	72	96	120
75	72 ± 14.9^{de}	84 ± 7.5^{abcd}	100 ± 0^{a}	100 ± 0.0^{a}	100 ± 0.0^{a}
97	0 ± 0^{g}	24 ± 7.5^{f}	76 ± 14.7^{cde}	100 ± 0.0^{a}	100 ± 0.0^{a}
R52	60 ± 0.0^{e}	80 ± 8.9^{bcd}	88 ± 8.0^{abcd}	100 ± 0.0^{a}	100 ± 0.0^{a}
S86	60 ± 11.0^{e}	72 ± 4.9^{de}	92 ± 8.0^{abc}	100 ± 0.0^{a}	100 ± 0.0^{a}
TK1	76 ± 0.0^{cde}	92 ± 0.0^{abc}	96 ± 0.0^{ab}	100 ± 0.0^{a}	100 ± 0.0^{a}
P value	< 0.001	< 0.001	< 0.001	> 0.001	>0.001

Means within a column with the same letters are not significantly different

Table 4.3 Mortality of 3rd instar larvae of *Tuta absoluta* over time at 200 concentration of entomopathogenic nematode isolates (Mean ± SEM)

EPN isolates	Exposure time (h)				
	24	48	72	96	120
75	52 ± 4.9^{ef}	88 ± 8^{abc}	96 ± 4^{ab}	100 ± 0.0^{a}	100 ± 0.0^{a}
97	0 ± 0.0^{g}	40 ± 8.9^{f}	80 ± 6.3^{bcd}	100 ± 0.0^{a}	100 ± 0.0^{a}
R52	64 ± 11.7^{de}	96 ± 4.0^{ab}	100 ± 0.0^{a}	100 ± 0.0^{a}	100 ± 0.0^{a}
S86	56 ± 16.0^{ef}	92 ± 4.9^{abc}	96 ± 4.0^{ab}	100 ± 0.0^{a}	100 ± 0.0^{a}
TK1	56 ± 7.5^{ef}	76 ± 7.5^{cd}	88 ± 8.0^{abc}	100 ± 0.0^{a}	100 ± 0.0^{a}
P value	< 0.001	< 0.001	< 0.001	> 0.001	>0.001

Means within a column with the same letters are not significantly different

Table 4.4 Mortality of 3rd instar larvae of *Tuta absoluta* over time at 250 concentration of entomopathogenic nematode isolates (Mean ± SEM)

EPN isolates	Exposure time (h)				
	24	48	72	96	120
75	40±8.9^{f}	76±7.5^{bcd}	96±4.0^{a}	96±4.0^{a}	100±0.0^{a}
97	0±0.0^{g}	40±12.7^{f}	68±12^{cde}	100±0.0^{a}	100±0.0^{a}
R52	52±10.2^{ef}	92±4.9^{ab}	100±0.0^{a}	100±0.0^{a}	100±0.0^{a}
S86	56±9.8^{ef}	88±8.0^{ab}	96±4.0^{a}	100±0.0^{a}	100±0.0^{a}
TK1	64±4.0^{de}	68±4.9^{cde}	84±9.8^{abc}	100±0.0^{a}	100±0.0^{a}
P value	< 0.001	< 0.001	< 0.001	> 0.001	>0.001

Means within a column with the same letters are not significantly different

4.3.4 Effect of entomopathogenic nematode isolates concentration on 2nd and 3rd instar larvae of *Tuta absoluta*

In the control experiment, there was no mortality observed over 120 h. At concentration 100, all the selected EPN isolates caused mortality >50 % on 2nd and 3rd instar larvae. The TK1 caused highest % mortality of 91.2±3.67 and 90.4±3.67 respectively on 2nd and 3rd instar larvae. Lowest % mortality of 59.2±8.37 and 60±6.43 was observed in EPN isolate 97 (Fig 4.3 A). There was no significant difference (P>0.001) between % mean mortality in 2nd and 3rd instar larvae of *T. absoluta* by each EPN isolate (Appendix 5).

All the EPN isolate caused >60 % mortality in the two instars at concentration 150. Isolate TK1 had the greatest % mean mortality of 95.2±2.09 and 92.8±3.24, while isolate 97 had the least mean of 60±8.869 and 63.2±7.544% in both larval stages (Fig 4.3 B). There was no significant difference (P>0.001) between % mean larval mortality among the EPN isolates (Appendix 6).

Similarly, all the EPN isolates registered >60% mortality in the two instars at concentration of 200. Isolate R52 recorded highest % mortality of 84.8±4.8 and 92±3.65 on 2nd and 3rd instar respectively. It was followed by isolate S86 with mean larval mortality of 87±3.98 and

89±4.63 (Fig 4.3C). Isolate 97 was the least infective among the EPN isolates with % mortality of 64±8.0 and 64±8.16. There was no significant difference (P>0.001) between 2nd and 3rd instar mean mortality among the EPN isolates (Appendix 7).

Percent mortality of >60% was recorded in the two larval stages at concentration 250 by all isolates. Isolate R52 had the highest % mortality of 87.2±4.45 and 89±4.33 followed by TK1 with % mortality of 86.4±3.6 and 83.2±3.74. Isolate 97 recorded least % mortality of 63.2±7.37 and 61.6±8.4 and 63.2±7.37 (Fig 4.3 D). There was no significant difference (P>0.001) between 2nd and 3rd instars % mortality in all EPN isolates (Appendix 8).

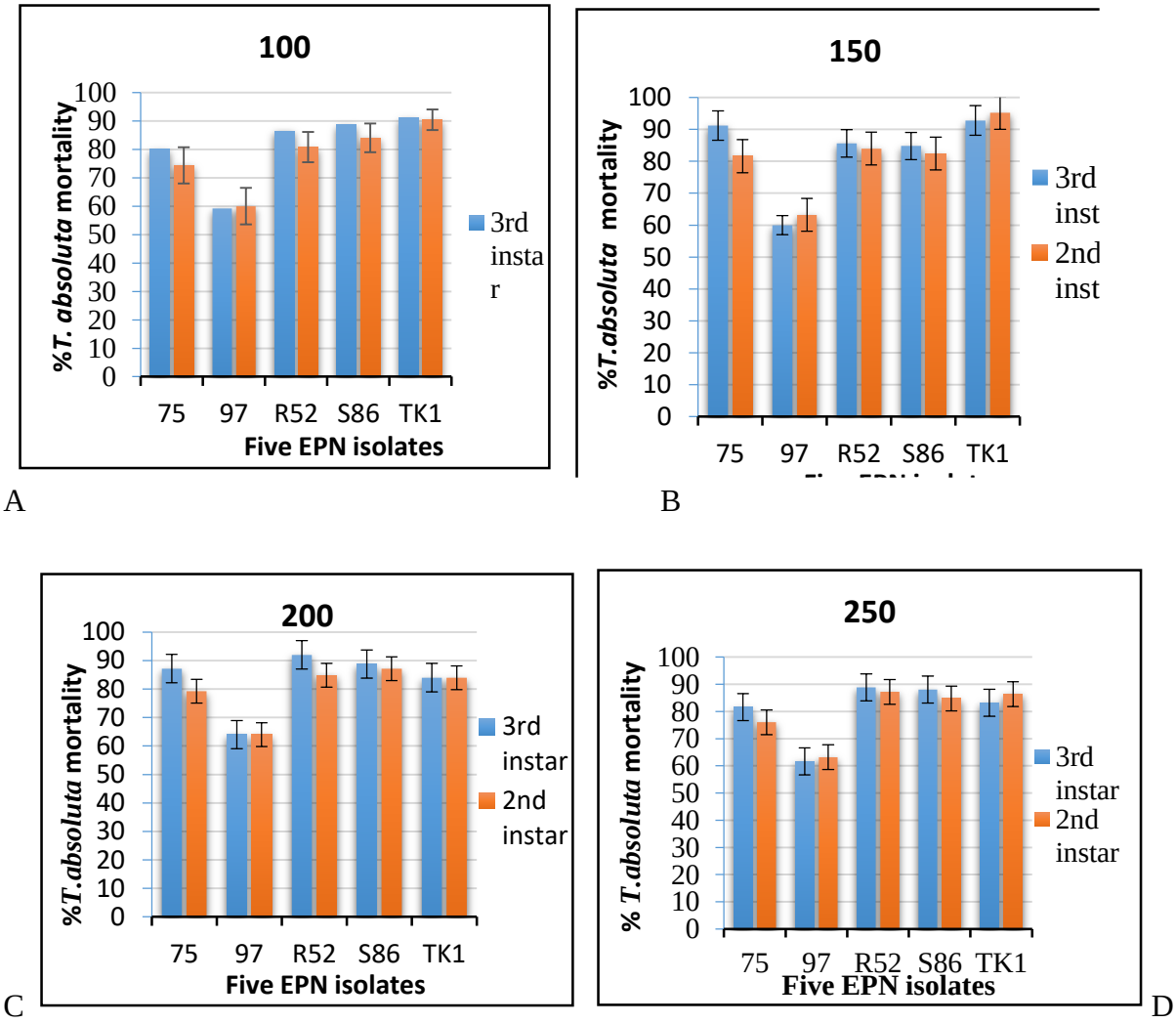


Figure 4.3 Infectivity of entomopathogenic nematode isolates on 2nd and 3rd larvae of *Tuta absoluta* at different concentrations (A 100, B 150, C 200 and D 250)

4.3.5 Effect of entomopathogenic nematode isolates on mortality of 2nd and 3rd instar larvae of *Tuta absoluta* against time

At 24 h all the EPN isolates caused mortality on *T. absoluta* apart from isolate 97 on the 3rd larval instar. Isolate 97 caused least % mortality in both larval instars (0±0; 9.6±2.99) at the 24 h. Isolate TK1 was leading with mean mortality of 52±3.35% and 51.2±4.08%. There was no significant difference (P>0.05) between mortality of the two larval stages by each isolate at 24 h (Table 4.5; Appendix 9).

Isolate 97 recorded lowest mean mortality of 28.8±4.63% and 33.6±4.12% in 3rd and 2nd instars at 48 h. Isolate R52 had the highest mortality of 71.2±0.80% only on 3rd instar and isolate TK1 with a mean of 68.8±3.88% on 2nd larval instar. Apart from isolate 97 all the other EPNs caused >50% mortality. There was no significant difference (P>0.05) in mortality of the two instars by each EPN at 48th h (Table 4.6; Appendix 10).

Table 4.5 Mortality of 2nd and 3rd instar larvae of *Tuta absoluta* at 24 h (Mean±SEM)

	T. absoluta larval stage		
Isolate	3rd	2nd	P-Value
Control	0±0[a]	0±0[a]	>0.05
75	38.4±2.0[b]	29.6±3.9[b]	
97	0 ±0[a]	9.6±2.99[b]	
R52	44.8±1.5[b]	36.8±3.4[b]	
S86	46.4±5.3[b]	42.4±4.8[b]	
TK1	52±3.4[b]	51.2±4.1[b]	

Means within a column with the same letters are not significantly different

Table 4.6 Mortality of 2nd and 3rd instar larvae of *Tuta absoluta* at 48 h (Mean±SEM)

	Larval stage		
Isolate	3	2	P-Value
Control	0±0[a]	0±0[a]	>0.05
75	66.4±3[b]	56±5.7[b]	
97	28.8±4.6[b]	33.6±4.1[b]	
R52	71.2±0.8[b]	64.8±1.9[b]	
S86	68±1.8[b]	61.6±2.9[b]	
TK1	65.6±1.6[b]	68.8±3.9[b]	

Means within a column with the same letters are not significantly different

Isolates R52, TK1, S86 and 75 had mean mortality >65% in both larval instars. The EPN 97 caused least mortality (57.6±3.9 and 54.4±5.88%) though mortality was >50% at 72nd h. There was no significant difference (P>0.05) in mortality of the two instars by each EPN isolate at 72nd h (Table 4.7; Appendix 11).

All the EPN isolates caused >70% mean mortality on both instars at 96th h. Isolates R52, TK1, and S86 had the highest mortality of >80% with isolate 97 recording the least mortality (78.4±0.98 and 73.6±1.6%). There was significant difference (P<0.05) in mortality by EPN isolate 97 on 3rd and 2nd larval only (Table 4.8; Appendix 12).

Table 4.7 Mortality of 2nd and 3rd instar larvae of *Tuta absoluta* at 72 h (Mean±SEM)

	T. absoluta larval stage		
Isolate	3rd	2nd	P-Value
Control	0±0[a]	0±0[a]	>0.05
75	76.8±1.5[b]	68.8±3.7[b]	
97	57.6±3.9[b]	54.4±5.9[b]	
R52	76.8±1.5[b]	75.2±1.5[b]	
S86	76±1.3[b]	74.4±2.0[b]	
TK1	73.6±2.04[b]	76±2.2[b]	

Means within a column with the same letters are not significantly different

Table 4.8 Mortality of 2nd and 3rd instar larvae of *Tuta absoluta* at 96 h (Mean±SEM)

	T. absoluta larval stage		
Isolate	3rd	2nd	P-Value
Control	0±0[a]	0±0[a]	< 0.05
75	78.4 ±0.9[b]	77.6±1.6[b]	
97	78.4±0.9[b]	73.6±1.6[c]	
R52	80±0[b]	80±0[b]	
S86	80±0[b]	80±0[b]	
TK1	80±0[b]	80±0[b]	

Means within a column with the same letters are not significantly different

Percent mortality of 80% was realized by isolates S86, TK1 and R52 on both larval instars apart from isolates 75 and 97 that had a mean of 79% on the 2nd larval instar. There was no significant difference (P>0.05) in mortality by all the EPNs on 3rd and 2nd larval instar in the 120thh (Table 4.9; Appendix 13).

Table 4.9 Mortality of 2nd and 3rd instar larvae of *Tuta absoluta* at 120 h (Mean±SEM)

	T. absoluta larval stage		
Isolate	3rd	2nd	P-Value
Control	0±0[a]	0±0[a]	>0.05
75	80±0[b]	79.2±08[b]	
97	80±0[b]	79.2±08[b]	
R52	80±0[b]	80±0[b]	
S86	80±0[b]	80±0[b]	
TK1	80±0[b]	80±0[b]	

Means in a columnwith the same letters are not significantly different

4.4 DISCUSSION

From this study, the results revealed that the five indigenous EPNs were infective against *T. absoluta* larvae as they caused larval mortality and infective juveniles recovered. This conforms to observations made by Gozel *et al.*, (2015) and Zolfagharia *et al.*, (2016), on infectivity of EPNs, against Diamond backmoth (DBM) larvae and *T. absoluta* larvae. According to Labaude and Griffin, (2018), EPNs may not be infective in some insects due to host resistance making it hard for nematodes to develop and reproduce within the host. It is suggested that the production of proteolytic enzymes from the symbiotic bacteria helps to overcome the problem of host resistance thus making it possible for EPNs to reproduce in insect cadavers. In this study, *T. absoluta* was susceptible thus did not have resistance against the study isolates, R52, S86, 75, 97 and TK1. The ability of EPNs to infect and reproduce within the host pest ensures their persistence and recycling ability in the natural environment. These are critical cosiderations for effective use of EPNs in pest control and commercial production efficiency (Kalia *et al.*, 2014).

Isolate TK1 was found to be infective as it caused mortality of between 60 and 90% to the 2nd and 3rd larvae of *T. absoluta* at low concentrations (100 and 150 IJs) in the first 48 h of

exposure. Isolate 97 caused lowest mortality of 0-48% at the same exposure time and concentration though infectivity increased with concentration and exposure time. Pesticides with fast knock down effect at low concentration reduces crop damage and is economical to use. The EPN isolates S86, 75 and R52 were found to cause variable infectivity of *T. absoluta* and they probably belong to the generalist category of EPNs (Dillman *et al*., 2012; Labuade and Griffin, 2018). Effectiveness of EPNs in pest management depends on, range of hosts, foraging ability and matching of species among others (Lacey and Georgis, 2012; Garcia-del-Pino *et al*., 2013; Hoctor, 2013; Nikdel and Niknam, 2015).

Results indicated that concentration of 200 at 72nd h resulted in the highest pest mortality in isolates TK1, S86, 75, 97 and R52. Thus a positive relationship between EPN isolates concentration and larval mortality was observed. The positive relationship was attributed to more EPNs penetrating the *T. absoluta* larvae leading to increased mortality. Kalia *et al*., (2014), reported that EPNs reproduction in host pest contribute to recycling ability which is key to effective pest control, commercial production efficiency and for their crucial survival in the environment. Screening for infectivity of EPN species or strain against a specific insect pest is a crucial step toward successful biocontrol strategy. There was a decrease in mortality of *T. absoluta* at dose 250, probably due to overcrowding of EPN isolate in the Petri dish and increased competition for entry into the host body, leading to their death hence low host infection.

The percentage mortality of *T. absoluta* increased with exposure period in all the EPN isolates. This finding disagree with Archana *et al.*, (2017), who reported decreased and lack of mortality in house fly in poultry manure infected with *Heterorhabditis* and *Steinernema* EPN species. Larval mortality was least at 24 h and highest at 96 h but differed among EPN isolates. For instance, isolate 96 caused least mortality (0-20%) in the 24th h of exposure but

reached 100% by the 120th h thus its effectiveness is dependent on exposure time. Infectivity of EPN differ with exposure time (Zolfagharian *et al.*, 2016).

Mortality of *T. absoluta* larvae differed among EPN isolates. Isolate 97 in terms of EPN type caused lowest mortality in both larval stages compared to all the other isolates regardless of concentration and exposure time. Different EPN species or strains behave differently toward host pest. This is in relation to mode of attack, entry point in to the pest, pathogenicity of the symbiotic bacteria harboured and the class of pest being infected (Kalia *et al.*, 2018; Labaude and Griffin, 2018). The EPN species contribute to the ability of IJs to penetrate the host whether it is through body openings or abrasion through the cuticle in case of *Heterorhabditis*. In addition, difference in infectivity of EPNs, is dependent on toxins and enzymes of symbiotic bacterium (Lacey and Georgis, 2012; Karimi and Salari, 2015).

4.5 CONCLUSION AND RECOMMENDATIONS

The study demonstrated that the five indigenous EPN isolates have potential in management of *T. absoluta* because the selected EPN isolates were infective to the 2nd and 3rd larvae of *T. absoluta*. Isolate TK1 was found to be the most infective to *T. absoluta* larvae among the tested EPN isolates.

From this study, it is recommended that, infectivity of the five EPN isolates on *T. absoluta* eggs and pupal stages be evaluated and identification of these native EPN isolates to species level to be carried out. Also, screen house and field trials to be conducted to verify the laboratory results.

CHAPTER FIVE

MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF ENTOMOPATHOGENIC NEMATODE ISOLATE TK1

5.1 INTRODUCTION

Steinernematid and heterorhabditid nematodes are lethal to economically impotant agricultural pests (Puza *et al.*, 2015; Shapiro-Ilan *et al.*, 2015). These nematodes infect and kill host insect in 24-72 h. Occurrence of entomopathogenic nematodes (EPNs) has been reported worldwide but their biological potential has not been exploited fully which has led to the interest for search and identification of suitable species (Nguyen *et al.*, 2007; Kalia *et al.*, 2014; Tumialis *et al.*, 2016). These EPNs exhibit differences in host range, infectivity, reproductive potential, commercial production and formulation. According to Puza *et al.*, (2015), about 90 *Steinernema* species have been described worldwide of which 15 species are from Africa. Finding and identifying indigenous EPNs population and species adapted to local climatic conditions is believed to be a crucial step for effective biological pest management which is an integral part of integrated pest management (IPM) (Salvadori *et al.*, 2012; Kalia *et al.*, 2014; de Brida *et al.*, 2017). Majority of EPN species have not been described and named taxonomically. However, tools for EPNs identification have been developed including body features and DNA sequence linked to morphological traits (Yan *et al.*, 2016).

Morphometric and morphological data commonly based on male tail, size and shape of spicules, body size, presence and absence of mucron and lateral lines of infective juveniles are crucial for preliminary grouping of EPNs (Nguyen, 2007; Hatting *et al.*, 2009). This preliminary grouping of EPNs though important is limited in that the value/ratios vary greatly hence need for alternative more precise identification technique including molecular method (Nguyen, 2007; Darissa and Iraki, 2014; Tumialis *et al.*, 2016).

Development of molecular applications has aided nematode species description and establishment of genetic relationship through phylogenetic reconstruction. This include analysis of internal transcribed spacer regions (ITS), partial 28S and 18S regions (D2 and D3) of the ribosomal DNA (rDNA) (Cimen *et al.*, 2016; Kaushik and Chaubey, 2016). Laboratory screening for infectivity of EPN isolates S86, R52, 75, and 97 and TK1 against *Tuta absoluta* confirmed TK1 as the most pathogenic isolate. The aim of the study was to characterize the most infective EPN isolate (TK1) against *Tuta absoluta* larvae using morphometrical, morphological features and molecular methods.

5.2 MATERIALS AND METHODS

5.2.1 Isolation and culturing entomopathogenic nematode isolate TK1

The EPN TK1 was isolated from soils in an avocado orchard at Kenya Agricultural and Livestock Research (KALRO) Kandara (Latitude -0° 99.65′ South, Longitude 37° 07.54′ East and altitude 1553m above the sea level). The EPN isolate was reared using insect baiting method as described by Bedding and Akhurst, (1975) and Nguyen *et al.*, (2007), where 250 g of soil was collected and 15 pre pupa stage of Greater wax moth (*Galleria mellonella*) placed on the soil in a bowl. The samples were stored at room temperature of 25±2 °C and inspected for larval mortality every 24 h. The *G. mellonella* cadavers were collected and examined for colour and presence of nematodes. The isolate culture named as TK1 isolate was established from infective juveniles (IJs) harvested using modified White trap method (use of white cotton cloth instead of filter paper) (White, 1927).

5.2.2 Temporary and permanent specimen preparation

Cadavers of *Galleria mellonella* infected with EPN isolate TK1 were surface sterilized in 70% ethanol, rinsed in distilled water and transferred into Ringer's solution where they were dissected. The required 20 fresh live 3rd stage IJs of isolate TK1 were individually picked

under dissecting microscope as they emerged from cadavers 5-7 day after infection. Also, a total of 20 live males of isolate TK1 were obtained from infected *G. mellonella* cadaver 3 days after baiting.

The isolate infective juveniles (IJs) and males were heat killed in a water bath at 50-60 °C for 3min. The specimen was fixed in 2-3 drops of double strength Triethanoalamine formalin (TAF) fixative (14 ml of 40%, of formaldehyde + 4 ml triethanolamine in 82 ml distilled water) (Courtney *et al.*, 1955). After 48 h, temporary mounts that were prepared were used within a week. Permanent mounts were made according to Seinhorst, 1959; Ryss, 2017). The fixed nematodes were placed in an excavated glass block and 7 ml of Solution 1 (20 parts of 95% ethanol and 1part of glycerine in 79 parts of distilled water) added. The glass block with samples was slowly dried by evaporation in a desiccator stuffed with cotton soaked in 95% alcohol. The glass block was placed in an oven at 35°C for 12 h after which it was flooded with Solution 2 (95 parts of 95% ethanol and 5 parts of glycerin); put in the oven at 40°C for 4 h after which the nematodes were mounted on microscope slides. The cover slip was supported and sealed with nail vanish. Morphological features considered for morphometrics were, nematode body length (L), maximum body diameter (MBD), anal body diameter (ABD), length tail (TL), distance from anterior end to oesophagus/pharynx (ES), spun from anterior end to excretory pore (EP), Hyaline (H) and in males, spicule length (SL), gubernaculum length (GL), presence and absence of mucron was considered (Nguyen, 2007; Mwaniki *et al.*, 2009; Ikoyi, 2012; Nguyen, 2017). Data on morphometric and images showing various nematode features were taken using compound microscope Leica Suit, DM 750 (LAS EZ Version 3.3.0 Build.181), fitted with digital color microscope camera LEICA EC4 (Leica Microsystems Switzerland Ltd). Other data on morphometric ratios and percentages included,

a=L/MBD b=L/ES c=L/T c′=T/ABD D%=EP/ES*100 E%EP/T*100

GS%=GL/SL*100 SW%=SL/ABD*100 H%=H/TL*100

5.3 Molecular identification of ITS region of isolate TK1

5.3.1 Specimen preparation

The *Galleria mellonella* larvae were infected with isolate TK1 and those that were tan in colour, spongy and odourless selected on the fifth day. The cadavers were surface sterilised in 70% alcohol. They were rinsed in distilled water and transferred to Ringer's solution (7.2 g NaCL, 0.37 KCL and 0.17 g $CaCL_2$ all dissolved in a litre of distilled water). The cadavers were then dissected in Ringer's solution to obtain fresh first generation gravid females. The females were preserved in 50 µl of 95% alcohol and stored at 4°C for later use.

5.3.2 Genomic DNA extraction

Genomic DNA was extracted from EPN isolates TK1 according to modified Razia *et al.* (2011) and Caoili *et al.*, (2017) protocol. Before DNA extraction the sample obtained from isolate TK1 (5.3.1) were rehydrated in distilled water overnight (12 h). Single female was put in 50 µl of lysis buffer, down spinning done and crushed using plastic pestle. The pestle was washed off with 50 µl of lysis buffer to remove any sample from the surface. Protenease K (50 µl) was added into and the samples incubated for 30 min at 37°C and transferred into water bath overnight (12 h) at 65°C.

Phenol-chloroform-isoamyl alcohol (200 µl) was added and the mixture centrifuged at 12000 rpm for 10 min. The upper layer was pippeted, 200 µl of chloroform-isoamyl alcohol added and the mixture centrifuged at 12000 rpm for 10 min to pellet the DNA. The solution's upper layer was pippeted into new 1.5 ml Eppendorf tubes and 200 µl of 96% alcohol together with 40 µl of 3M of Ammonium acetate added. The mixture was precipitated for 3 h at -20 °C after which it was centrifuged for 10 min at 12000 rpm. The solution was decanted and the eppendorfs inverted to dry off the remaining alcohol and ammonium acetate from the DNA pellets for 1 h at 25 °C room temperature. The DNA was re-suspended in 100 µl of sterile

double distilled water hence no need for DNA purification. The DNA obtained was quantified and purity analysed on a spectrophometer and stored at -40 °C for later use.

5.3.3 Polymerase Chain Reaction (PCR) Reaction of ITS-rDNA

The PCR was done to amplify the ITS region of the local EPN isolate TK1 in 12.5 µl of 10x of PCR master mix (Bio lab, England), 10x of 1.0 µl forward primer (TW81) 5'-GTTTCCAGTAGGTGAACCTGC-3' 10x of 1.0 µl reverse primer (AB28) 5'-ATATGCTTAAGTTCAGCGGGT-3' (Joyce *et al.*, 1994), 10x of 7.5 µl sterile double distilled water and 3.0 µl template, all contents set on ice. In PCR tubes, 22 µl of master mix was dispensed and 3 µl of each DNA sample was added to make a total of 25 µl. Down spinning of the mixture was done and placed in a thermocycler (ProFlex PCR System Applied biosystem) temperature set at 94 °C for 5min, 94°C for 1min, 55 °C for 1 min, 72°C for 2 min and 72 °C for 5 min all at 35 cycles per min. The target region for rDNA amplification by Polymerase Chain Reaction (PCR) was ITS -5.8s- ITS2 which varies among different EPN species.

5.3.4 Gel electrophoresis

The electrophorese gel was prepared by adding 150 ml of Tris Acetate EDTA (TAE), buffer to 1.5 g of agarose agar. The mixture was heated to boil in an oven at 30 sec intervals for 1min 30 sec and, 3.5 µl of ethidium bromide added. The mixture casted into the gel tank and left 30 min to solidify after which the gel was placed in the electrophoresis tank. The PCR products, 5 µl were mixed with 3 µl of loading dye and dispensed into the wells. Distilled water was used as negative control and 1500 base pair ladder was loaded in the 1st well. The electrophoresis was run at 100 volts for an hour. Presence or absence of DNA was confirmed by appearance of light bands in the lanes loaded with amplified PCR products. The success of the amplification was determined by the brightness of the bands, the brighter the band, the more successful the amplification. A DNA ladder (Biolabs, England), was used to infer the

band sizes of the amplified samples and a gel picture taken using a Kodak UV GL200 camera. The PCR products were sent for sequencing to Microgen lab in Korea.

5.3.5 Sequence analysis of entomopathogenic nematode isolate TK1

The sequence of isolate TK1 was analyzed using bioinformatics tools as follows: editing of the sequence was done using BioEdit v 7.0.5 Sequence Alignment Editor (Hall, 1999). Multiple Sequence alignment was done using MUSCLE on SeaView version 4 Alignment and analysis program (Edgar, 2004; Gouy *et al.*, 2010). A similarity search of the deduced consensus sequence of EPN from the Genbank database was then done using Basic Local Alignment Search Tool (BLASTn) at https://blast.ncbi.nlm.nih.gov.) (Altschul *et al.*, 1990; Altschul *et al.*, 1997). The best seventeen BLAST hits obtained of known EPNs including *S. karii* indigenous to Kenya were retrieved and their sequences aligned using the SeaView alignment tool.

5.3.6 Phylogenetic analysis of entomopathogenic nematode isolate TK1

The retrieved ITS sequences were used in phylogenetic tree reconstructed using Neighbour-Joining, Distance method on SeaView version 4 (Edgar, 2004; Gouy *et al.*, 2010) for automatic tree generation. Bootstrap analysis was performed at 1000 replications at 70% threshold for relatedness. The tree provided information on organism clustering of the sequences in order to augment BLASTn search information.

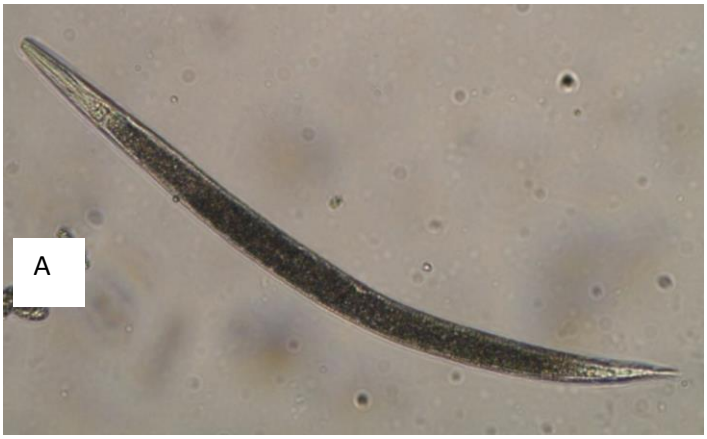
5.4 DATA ANALYSIS

The morphological data was analysed using Excel 2013 version, means and ratios calculated. The EPN isolate TK1 sequence and phylogeny analysis were done using BioEdit v.7.5. and SeaView v.4. bioinformatics programmes.

5.5 RESULTS

5.5.1 Morphology of third stage infective juveniles of entomopathogenic nematode isolate TK1

Tan /brown colour was observed in *Galleria mellonella* cadavers infected with isolate TK1. The morphometric data for the 3rd stage infective juveniles (IJs) of isolate TK1 are summarized in Table 5.1. Heat killed juveniles were almost straight or slightly C-shaped. The IJs had an elongate body (834.54±87.36 µm) length twice that of males (1781.33±195.07 µm) while maximum body diameter (MBD) was two times and half longer than of the males MBD (113.46±20.87 µm). The IJs body tapered gradually anteriorly and posteriorly. The anterior end was blunt. The IJs had a short hyaline 20.47±3.33µm (13.45-24.95 µm long that was half the tail length (Fig 5.1 A, B and C; Table 5.1; Appendix 14).



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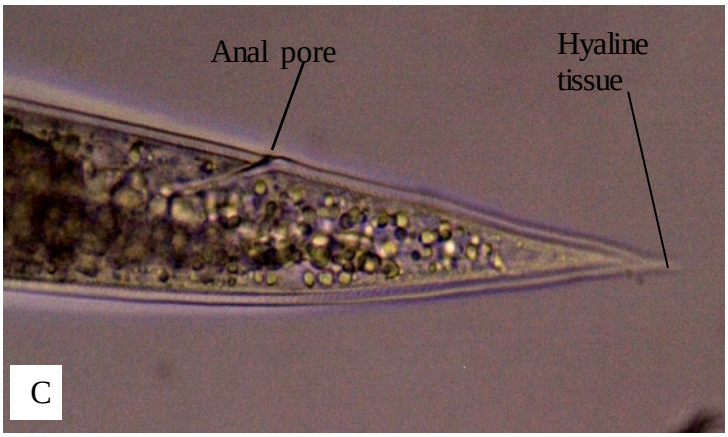

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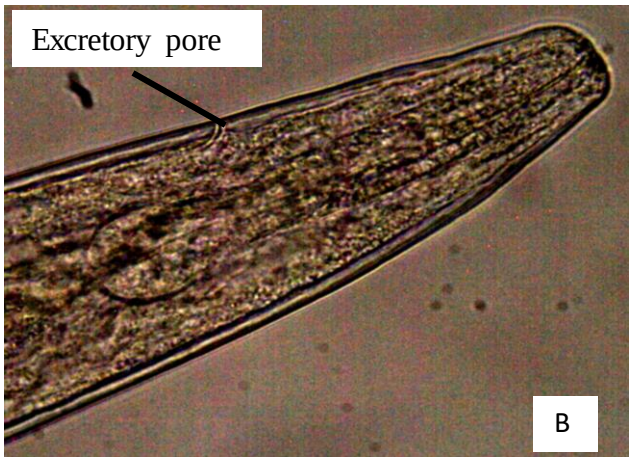
Photo: Ngugi, CN. 2019

Figure 5.1 Infective juveniles of entomopathogenic nematode isolate TK1; (A) Slightly C shaped, (B) Tapered but blunt end anterior terminus and (C) Tail region with anal pore and hyaline tissue

5.5.2 Morphology of first generation Males of entomopathogenic nematode isolate TK1

The heat killed males assumed a J shape, tapered anteriorly, posterior end strongly ventrally curved and average body length of 1781.33±195.07 µm. The anterior terminus was flat and pharynx more or less cylindrical. Procorpus and metacorpus cylindrical and same width but the isthmus was indistinct. The excretory pore was 106.37±13.77 µm more posterior to the mouth, $^{2}/_{3}$ of pharynx region and anterior to basal bulb. The basal bulb was rounded with a narrow base. The spicule was 82.05±13.94 µm long, large with rounded anterior end, strongly curved, paired and golden brown in colour. It was also slightly curved, the head (manubrium) wider than long, short shaft (calomus) present, velum wide and the spicule tip conoid. The gubernaculum was about half (45.28±5.89 µm) the spicule length. It was curved ventrally. The tail was short, an average 19.97±2.84 µm long, rounded blunt end and mucron absent. The tail was slightly longer than half anal body diameter (Fig 5.2 A, B and C; Table 5.1; Appendix 15). The male had 3pairs of subterminal postcloaca papillae, single pappilae, 5 sub ventral and adanal pair posterior to cloaca. Testis reflexed ventrally and monarchic.

X40



X100

X100

Photo: Ngugi, CN. 2019

Figure 5.2 (A) posterior strongly J shaped male, (B) anterior region with excretory pore, and (C) Tail region with spicule and gubernacul3m

Table 5.1 Morphometrics of 3rd stage infective juveniles and 1st generation males of entomopathogenic nematode isolate TK1

Character	Infective juveniles	First generation males
n	20	20
L	834.54±87.36 (658.60-986.89)	1781.33±195.07 1296.62-2096.87
a	17.79±1.06 (16.26 -19.70)	15.98± 2.22 12.43-19.45
b	7.98±1.57 (5.90 - 12.15)	15.07±2.19 (10.76-19.44)
c	15.94±2.30 (11.86 - 20.45)	91.35±18.60 (57.17-125.56)
c′	2.45±0.33 (1.82 - 2.98)	- -
EP	81.23±7.06 (62.66-95.57)	106.37±13.77 84.51-140.13
MBD	46.92±4.23 (38.98- 55.30)	113.46±20.87 (90.21-162.74)
ES	106.57±12.78 (70.98-118.86)	119.39± 13.57 (93.10-150.77)
T	53.22±8.35 (40.30-71.51)	19.97±2.84 (14.20-25.70)
ABD	21.85±2.83 (16.50-25.97)	33.23 ±4.87 (25.62-45.03)
H	20.47±3.33 (13.45-24.95)	- -
SPL	- -	82.05±13.94 (57.86-128.30)
GL	- -	45.28±5.89 (34.48-54.87)
SW%	-	73.60±13.01 (50.90-89.53)
GS%	- -	56.30±10.32 (36.98-86.28)
D%	77.59±14.00 (61.80-114.09)	89.18± 6.49 (77.70-100.78)
E%	291.22±34.69 (219.02-363.80)	542.72±105.13 (424.35-785.40)
H%	2.47±0.45 (1.65-3.37)	- -

n=No of specimen; a=L/MBD; b=L/ES; c=L/T; c′=T/ABD; D%=EP/ES*100; E%EP/T*100; GS%=GL/SL*100; SW%=SL/ABD*100; H%=H/TL*100; L= body length; MBD=maximum body diameter, ABD=anal body diameter; T=length tail; ES=pharynx length; EP=excretory pore; SL= spicule length, GL=gubernaculum length; -, Data not available

5.5.3 Comparative morphometrics of infective juveniles of entomopathogenic nematode isolate TK1

The average body length (834.54±87.36 µm) was close to *S. ethiopiense* (898±50 µm). The b ratio and maximum body diameter (MBD) compared narrowly with *S. jeffreyense*, *S. ethiopiense* and *S. pwaniensis*. The c ratio of isolate TK1 (15.94±2.30 µm) was close to all the comparator juveniles. Isolate TK1 excretory pore (81.23±7.06 µm) differed distantly with *S. karii* (74±3 µm) but narrowly to *S. jeffreyense*, *S. ethiopiense* and *S. pwaniensis*. The tail length (53±8.35 µm) and hyaline (20.47±3.33 µm) were too short compared to the *S. jeffreyense*, *S. ethiopiense* and *S. pwaniensis* (Table 5.2).

Table 5.2 Comparative morphometrics of Infective juveniles of entomopathogenic nematode isolate TK1 with Mean±SD and ranges in µm

Character	Fresh Infective juveniles				
	TK1	*S. jeffreyense*[1]	*S.karii*[2]	*S.ethiopiense*[3]	*S.pwaniensis*[4]
n	20	20	20	20	20
L	834.54±87.36* (658.60-986.89)	926±83 (784–1043)	932±24 (876-982)	898±50* (768-1010)	978±75 (808-1131)
a	17.79±1.06 (16.26 -19.70)	26 ±3.2 20-35	28.5 -	27±2.6 (20-30)	25±1.4 (23-29)
b	7.98±1.57* (5.90 - 12.15)	7.0± 0.5* (6.1-7.9)	- -	6.4±0.3 (5.7-7.1)	7.0±0.4* (6.2-7.6)
c	15.94±2.30* (11.86 - 20.45)	12± 22.0 * (9.7-19)	12.6* -	12±0.6 (11-14)	11±0.7* (10-12)
c′	2.45±0.33* (1.82 - 2.98)	2.7± 0.4* (2.1-3.5)	- 3.4	3.2±0.2* (2.9-3.8)	3.8±0.5 (3.3-5.4)
EP	81.23±7.06* (62.66-95.57)	87± 9.3* (78-107)	74±3 (68-80)	78±4.6* (65-84)	86±4.7* (80-95)
MBD	46.92±4.23* 38.98- 55.30)	35 ± 4.4* (23- 43)	33±1.4 (31-35)	34±3.0 (32-43)	39±3.1* (32-45)
ES	106.57±12.78 (70.98-118.86)	133±7.2 (116-149)	- -	140±6.9 (127-153)	140±5.7 (130-151)
T	53.22±8.35 (40.30-71.51)	81±10.1 (50-96)	74±4.5 (64-80)	73±3.9 (64-80)	87±5.4 (75-95)
ABD	21.85±2.83* (16.50-25.97)	39±4.6 (19-39)	22±0.6* (64-80)	23±1.3* (19-24)	23±2.2* (17-27)
H	20.47±3.33 (13.45-24.95)	44±6.3 20-52	- -	38±3.7 (29-43)	46±4.7 (37-54)
D%	77.59±14.00* (61.80-114.09)	66±6.1* 57-85	- -	56±2.2 (51-58)	61±2.6 (57-67)
E%	291.22±34.69 (219.02-363.80)	109± 19 (86-169)	96 -	107±5.5 (91-116)	98±6.4 (83-110)
H%	2.47±0.45 (1.65-3.37)	43±6.3 (20-52)	-	52±3.0 (43-56	53±3.1 (49-60)

n=No of specimen; a=L/MBD; b=L/ES; c=L/T; c′=T/ABD; D%=EP/ES*100; E%EP/T*100; GS%=GL/SL*100; SW%=SL/ABD*100; H%=H/TL*100; L= body length; MBD=maximum body diameter; ABD=anal body diameter; T=length tail; ES=pharynx length; EP=excretory pore; SL=spicule length; GL=gubernaculum length; H=Hyaline;

Data from: [1]Malan, *et al*., 2016; [2]Waturu *et al*., 1997; [3]Tamiru, *et al*., 2012; [4]Puza *et al*., 2015; -Data not available; *Data in a row indicate close means

5.5.4 Comparative morphometrics of first generation males of isolate TK1

The body length, anterior distance to excretory pore, and spicule length (1296.62-2096.87 µm, 106.37±13.77 µm and 82.05±13.94 µm) of TK1 were close to that of *S. karii*, (1400-2400, 108±14µm and 83±4 µm). Ratios a= L/MBD and b=L/ES (15.98± 2.22µm; 15.07±2.19 µm) of isolate TK1 measured closely to *S. jeffreyense* (12± 1.7; 11±1.0 µm) and ratio b only with *S. pwaniensis* (12±1.3 µm). The gubernaculum length (45.28±5.89µm) compared narrowly with *S. ethiopense* (49±1.3). The D%=EP/ES*100 for EPN isolate TK1 was close (89.18±6.49 µm) to *S. pwaniensis* (85±8.0 µm). However, Isolate TK1 tail and anterior distance to excretory pore length varied distantly from the comparison species but a mucron was absent in all (Table 5.3).

Table 5.3 Comparative morphometrics of First generation males of entomopathogenic nematode isolate TK1 with Mean±SD and ranges in µm

	First generation males				
Character	TK1	*S. jeffreyense*[1]	*S. karii*[2]	*S. ethiopense*[3]	*S. pwaniensis*[4]
n	20	20	20	20	20
L	1781.33±195.07 1296.62-2096.87*	1634±130 (1740–1899)	1900±0.3[q] 1400-2400*	1081±40 (1028-1232)	2104±247 (1616-2586)
a	15.98± 2.22* (12.43-19.45)	12± 1.7* (9.9 –15.2)	13.7 [q] (11.7-17.1)	12±0.6* (11-13)	13±1.7* (11-16)
b	15.07±2.19* (10.76-19.44)	11±1.0* (9.4-13)	11.3[q] (9.0-13.3)	7.1±0.2 6.3-7.4	12±1.3* (9.6-15)
c	91.35±18.60 (57.17-125.56)	61±6.2 (49-72.5)	50[q] (42-70)	42±3.4 (35-49)	50±6.2 (38-66)
EP	106.37±13.77* 84.51-140.13	71± 10 (50-99)	108±14* (86-138)	88±1.7 85-92)	145±16 (113-172)
MBD	113.46±20.87 (90.21-162.74) *	139±22 (94-167) *	136±17 [q] (107-166)	92±48 (81-99)	158± 26 (109-196)
ES	119.39± 13.57 (93.10-150.77)	151±8.5 (136-165)	164±7 (146-187)	153±3.1 (149-161)	170±11 (152-192)
T	19.97±2.84 (14.20-25.70)	27±3.01 (21-33)	37±7.5 (22-48)	29±1.9 (25-33)	42±5.0 (30-56)
ABD	33.23 ±4.87* (25.62-45.03)	42±3.8* (35-49)	55±5 (43-66)	45±0.8 (43-46)	53±5.8 (43-65)
SPL	82.05±13.94* (57.86-128.30)	88±3.6 (79-95)	83±4* (73-91)	73±2.0 (69-77)	92±4.6 (80-97)
GL	45.28±5.89* (34.48-54.87)	57±2.8 (51-61)	57±6 (42-64)	49±1.3* (46-57)	60±2.2 (56-64)
SW%	73.60±13.01 (50.90-89.53)	215±27 (171-295)	151[q] -	164±5.8 154-175	178±19 (146-226)
GS%	56.30±10.32 (36.98-86.28)	65±2.57 (61-71)	70±1[q] (50-80)	67±2.1 (63-70)	65±2.9 (61-72)
D%	89.18± 6.49* (77.70-100.78)	47±8.4 (34-68)	66 (57-78)	57±1.6 (54-61)	85±8.0* (71-98)
E%	542.72±105.13 (424.35-785.40)	47±8.4 (34-68)	240[q] -	304±23 (264-357)	345± 51 (274-453)
Mucron	Absent	Absent	Absent	Absent	

n=No of specimen; a=L/MBD; b=L/ES; c=L/T; D%=EP/ES*100; E%=EP/T*100; GS%=GL/SL*100; SW%=SL/ABD*100; L= body length; MBD=maximum body diameter; ABD=anal body diameter; T=length tail; ES=pharynx length; EP=excretory pore; SL=spicule length, GL=gubernaculum length

[1]Malan, *et al*., 2016; [2]Waturu *et al*., 1997; [3]Tamiru, *et al*., 2012; [4]Puza *et al*., 2015; [q]after Nguyen and Hunt, 2007; * Data in a row indicate close means; Data not available

5.5.5 Electrophoresis of PCR product of entomopathogenic nematode isolate TK1

The electrophoresis gel image of amplified DNA of isolate TK1 was as in Fig 5.3. Sequence length of between 850-900 bp was obtained (Fig 5.3).

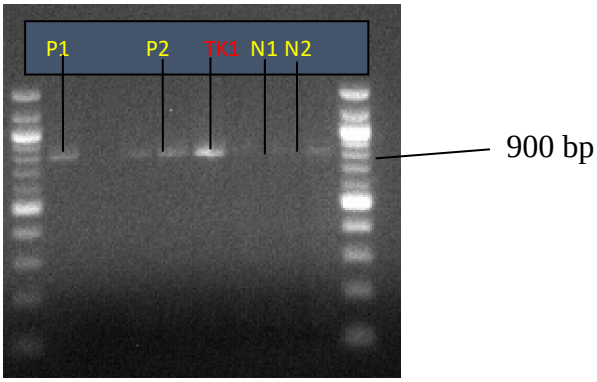


Photo: Ngugi, CN. 2019

Figure 5.3 Gel image of DNA from isolate TK1
P1and P2, positive controls (DNA from known EPN; N1and N2, negative controls (PCR Mastermix without DNA)

5.5.6 Sequence and phylogenetic analysis

The partial length of ITS of rDNA sequence of entomopathogenic nematode (EPN) isolate TK1 was 877 base pairs (bp) (Appendix 16). The BLAST results of ITS region had sequence maximum identity/similarity of 92%, 87%, 85%, 84%, 83% and 85%, with a EPN *Steinernema* sp. from Kenya (Genbank accession number AY230186.1), Sri Lanka, (AY230184.1), Tanzania (KT358812.1), *S. karii* from Kenya (AY230173.1), *S. ethiopense* (JN651414.1) from Ethiopia and *Steinernema* spp. (KT358811.1) from Tanzania respectively. The closest relative species is the afro-tropical *Steinernema* spp., AY230186 recorded from Kenya.

Phylogenetic tree constructed using 17 *Steinernema* species/strains selected from BLASTn search results, was based on maximum query coverage, homology, lowest E-Values and highest percent identity in relation to EPN isolate TK1 (Appendix 17). The tree showed trichotomy placing isolate TK1 together with selected known Eastern Africa *Steinernema*

spp. (*S. pwaniesis, S. ethiopiense, S. karii*). The isolate clustered in a clade sub-branch with *Steinernema* spp. from Kenya (AY230186.1) and Sri lanka (AY230184.1) with bootstrap value of 100% hence least divergent species from isolate TK1. The other close relative from Kenyan *S. karii* was in a different sub-branch, clustering with Ethiopian species (JN651414.1) *S. ethiopense*, JN651413.1 and JN651412.1). The rest of comparative *Steinernema* species formed a distinct clade with sub branches. The *Heterorhabditis safricana* (EF88006) was the most divergent species from isolate TK1 hence more of an out group among the comparator species (Fig 5.4).

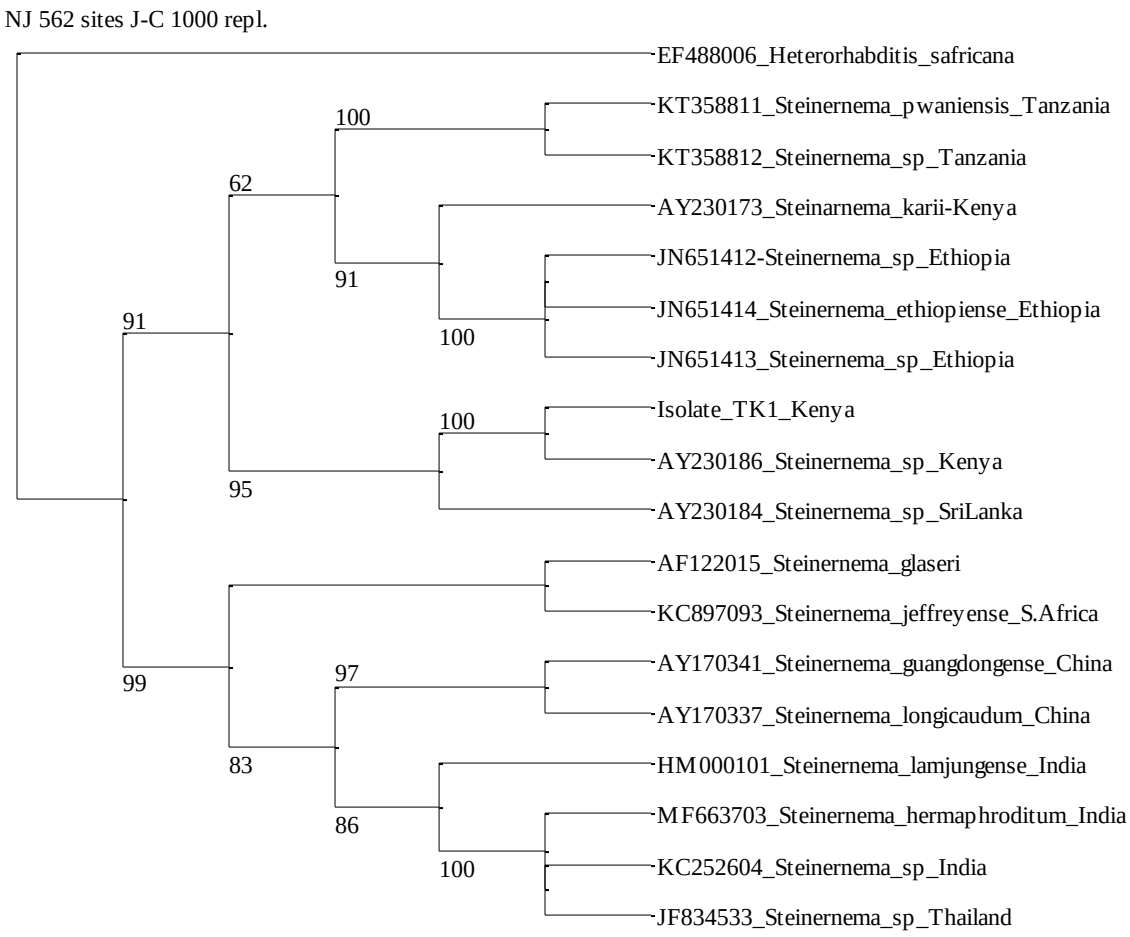


Figure 5.4 Phylogenetic relationship of entomopathogenic nematode isolate TK1

5.6 DISCUSSION

From this study, tan/brown colour was observed in *Galleria mellonella* cadavers infected with EPN isolate TK1 suggesting isolate TK1 belongs to genus *Steinernema*. Shades of brown colour are reported in *G. mellonella* cadavers infected with EPNs *Steinernema* species (Griffin, 2012). Results of pale brown colour in termites infected with *Steinernema karii* were reported by Wagutu *et al.*, (2017). The body length in infective juveniles (IJs) of EPN isolate TK1 corresponded with that of *Steinernema* sp. in feltiae group of EPNs. This result further supported possibility of EPN isolate TK1 to be a *Steinernema* species. Based on body length *Steinernema* species IJs are grouped into glaseri (>1000µm), feltiae (<1000 but >700µm), intermediate (<700 but >600µm), carpocapsae (<600µm) and bicornutum (with 2 horn like structures) group (Nguyen, 2017).

First generation males of EPN isolate TK1 were characterized by lack of a mucron on the tail tip, light golden brown colour of the spicule and heat killed males that were J shaped but strongly curved posteriorly almost spiral. These characteristic feature place EPN isolate TK1 closer to identified *Steinernema* species of EPNs. The absence of mucron and presence of brownish spicule in first generation males of EPNs *Steinernema karii, S. biddulphi* and *S. ethiopense* were reported by Waturu, (1998); Tamiru *et al*., (2012) and Cimen *et al*., (2016). In adition, Nikdel and Niknam, (2015) reported brownish spicule and monarchic and reflexed testis in EPN *Steinernema feltiae* from Iran.

The EPN isolate TK1 related morphometrically and morphologically closely to *S. karii, S. pwaniensis, S. ethiopense* and *S. jeffreynse* which are geographically African in origin. The IJs of isolate TK1 were distinct from comparative species *S. jeffreyense, S. karii* and *S. ethiopense* and *S. pwaniensis* by short pharynx, tail and high E% value. Molecular analysis placed EPN isolate together with *Steinernema* sp. from genbank but none gave 100% match, thus the isolate is suspected to be a new species. According to Nguyen, (2017), EPNs

Steinernema spp. are five groups namely; feltiae, glaseri**,** intermedium, carpocapsae and bicornutum. Based on molecular analysis, most of "feltiae" group members are found in "glaseri group. This include relatives of EPN isolate TK1; the *Steinernema karii* (Kenya), *S. pwaniensis* (Tanzania), *S. ethiopense* (Ethiopia) and *S. jeffreynse* (South Africa) all referred to as African *"*glaseri" (Waturu *et al.*, 1998; Malan *et al.*, 2016; Puza *et al.*, 2015). Further, phylogenic analysis revealed close relatives of EPN isolate TK1 outside Africa, *S. glaseri* (AF122015.1) Belgium; *S. guandlongense* (AY170341.1) China; *S. longicaudum* (AY170337.1) China; *S. lamjungense* (HM000101.1) India and *S. hermaphroditum* (MF663703.1) India; are in the "glaseri" group of EPNs (Nguyen, 2017).

5.7 CONCLUSION AND RECOMMENDATION

The EPN isolate TK1 is a Steinernematidae with close morphometric and morphology with *S. karii* (Kenya*), S. pwaniensis* (Tanzania), *S. ethiopense* (Ethiopia) and *S. jeffreynse* (South Africa), which are *Steinernema* species and African in origin. The EPN isolate TK1 is thus placed in *feltiae-glaseri* group of EPNs. The isolate was distinct from the three described species by its infective juveniles (IJs) and males mainly by the high E% value, shorter pharynx and tail. golden light brown colour of the spicule and a strong ventral posterior curvature almost spiral of heat killed males. The sequence of ITS region of rDNA and phylogenetic analysis supported identification of EPN isolate TK1 as a *Steinernema* species in the glaseri group of EPNs. The EPN isolate TK1 is suspected to be a new *Steinernema* species of EPN from Kenya.

From this study, it is recommended that, further morphological investigations be conducted using Scanning Electron Microscope (SEM) to ascertain and identify EPN isolate TK1 to species level.

CHAPTER SIX

ISOLATION AND IDENTIFICATION OF SYMBIOTIC BACTERIA FROM ENTOMOPATHOGENIC NEMATODE ISOLATE TK1

6.1 INTRODUCTION

Entomopathogenic nematodes (EPNs) in Steinernematidae and Heterorhabditidae families are soil inhabiting soft non-segmented roundworms and are lethal against economically important crop pests (Aiswarya *et al.*, 2017; Cruz-Martinez *et al.*, 2017). The free living and non-feeding stage of EPNs known as infective juvenile (IJ) infect an insect by penetrating through body orifices and cuticle. The *Steinernema* and *Heterorhabditis* are symbiotically associated with pathogenic bacteria species *Xenorhabdus* and *Photorhabdus* respectively (Ferreira and Malan, 2014; Kalia *et al.*, 2014; Abdolmaleki *et al.*, 2016; Salgado-Morales *et al.*, 2019). These bacteria belong to family Enterobacteriaceae in the gamma subdivision of proteobacteria (Sangeetha *et al.*, 2016; Salgado-Morales *et al.*, 2019). The *Xenorhabdus* bacteria inhabit bacterial vesicle of *Steinernema* while *Photorhabdus* are found in the gut of *Heterorhabditis* EPNs. Once the IJs invade an insect, the pathogenic bacteria are released from intestines into the hemocoel of the host insect (Sternberg and Dillman, 2012; Ferreira and Malan, 2014; Abdolmaleki *et al.*, 2016; Aiswarya *et al.*, 2017). The bacteria multiply as the EPNs nourish on them and insect tissue and reproduce killing the insect host within 24-48 h (Shapiro-Ilan *et al.*, 2015; Ulug *et al.*, 2015). The symbiont bacteria provide pathogenicity, degrade and breakdown host tissues and suppress immunity of the host during which the EPN reproduce and complete the growth cycle. The bacteria also produce antibiotics and enzymes in addition to toxins. The symbiont bacteria in the EPNs gut are referred to as phase I cells while variant forms are the phase II cells which are observable under laboratory experiments. The mutualism of bacteria and EPNs is vital as it inhibits development of resistance in the host pest (Proschak *et al.*, 2011; Poinar and Grewal, 2012; Sternberg and Dillman, 2012;

Kalia *et al.*, 2014). Different EPNs harbor specific bacterial symbiont which is believed to dictate the virulence of the vector nematode. Biochemical, phenotypic and DNA analysis have been used in the identification of bacteria. Molecular DNA/DNA hybridization methods and total protein profiling have been employed in symbiotic bacteria species identification. Recently, 16S rDNA sequencing and phenotypic evaluation have also been used in characterization and identification of new bacteria species (Salvadori *et al.*, 2012; Sangeetha *et al.*, 2016; Ahmed *et al.*, 2018). This study aimed at determining the identity of symbiotic bacteria associated with indigenous EPN isolate TK1, through biochemical and molecular methods.

6.2 MATERIALS AND METHODS

6.2.1 Isolation of bacteria from infective juveniles of entomopathogenic nematode isolate TK1 and haemolymph of *Galleria mellonella* larvae

Symbiotic bacteria from entomopathogenic nematode (EPN) isolate TK1 was isolated from infective juveniles (IJs) according to method described by Akhurst, (1980). From EPNs stock suspension, 50 µl of IJs were obtained and placed in petri dish. The IJs were surface sterilized in 1% Sodium hypochlorite for 15 min, rinsed twice in distilled water and lastly in Ringer's solution. The IJs were picked and crushed manually using a micropestle. A loopful of the merceratedmaterial was inoculated in Luria-Bertani (LB) broth (10 g Bacto-Tryptone, 5 g Bacto-Yeast extract and 10 g NaCl in a litre of distilled water) and plates with NBTA media. Ten replications were made for each media and incubated for 48 h at 35°C.

Galleria mellonella larvae were infected with EPN isolate TK1. The symbiotic bacteria associated with TK1 was isolated according to method described by Woodring and Kaya, (1988). Four to five days after infection tan brown, odourless and firm *G. mellonella* cadavers were selected, sterilized for 5 min in 70% alcohol, flamed and allowed to dry in a laminar airflow cabinet for 2 min. Using sterile forceps and scalpel a third segment from the

head was cut and the haemolymph streaked onto nutrient agar and NBTA plates using sterile wire loop. The plates were sealed and incubated for 48 h at 35°C for bacteria growth. Single bacteria colonies were then selected and streaked onto new plates and subcultured until colonies of same size and morphology were obtained. Bacteria culture was also established in LB broth. This was then incubated for 48 h at 35°C. For short term storage (three-four months) the bacterium was maintained on nutrient agar plates at 28°C and liquid broth at 4°C.

6.2.2 Bacterial isolate TK1 phenotypic and biochemical analysis on different media

The bacterial phenotypic and biochemical assessment were done according to Kumar *et al.*, (2011) method.

6.2.2.1 Gram Staining

A small drop of distilled water was placed on sterile slide. A loop full of the inoculum of bacteria isolate TK1 was scooped from colonies on NBTA media and spread thinly on the slide. The smear was heat fixed by passing the slide over a flame 3 times. The smear was placed on a staining rack where it was flooded with Crystal violet stain (2 g Crystal violet, 20 ml Ethyl alcohol, 0.8 g Ammonium Oxalate and 80 ml distilled water) for 1 min and then washed in distilled water. Gram's iodine (1 g Iodine, 2 g Potassium iodide and 100 ml distilled water) was added, let to stand for 1 min and washed off. The smear was decolourised using acetone (1:1 acetone 50 ml and 50 ml of 95% alcohol) till blue colour cleared. The smear was then counter stained with Safranin (0.34 g Safranin, 10 ml absolute alcohol and 90 ml distilled water) for 3 min, rinsed with distilled water, dried by air or blotting and presence or absence of primary stain observed under the microscope (Leica DM 500), at x100 objective with oil immersion.

6.2.2.2 Symbiotic bacteria growth on differential media

A loopful of pure bacteria isolate TK1culture was streaked on Nutrient agar (NA), NBTA and MacConkey media (3.6.1). Uninoculated plates were used as negative controls (-ve). All the media plates were incubated for 3 days at 35 °C and observed for bacterial growth and colony characteristics (shape, colour, margins).

6.2.2.3 Glucose fermentation (Acid gas production) test

Acid and gas production test was done using purple glucose broth (0.015 g Bromcresol Purple, 10 g glucose, 5 g Sodium chloride and 1lt distilled water). Sterile glucose broth (10 ml) was dispensed in test tubes with Durham's tubes. The broth was inoculated with loopful of bacteria isolate TK1. The set up was incubated at 35 °C for 3 days and gas production and colour change observed.

6.2.2.4 Motility test

This was done using semi solid NA media which had been prepared at half strength (3.6.1). The media was inoculated by single stab at the centre half depth the tube using a sterile straight wire. The inoculated media was incubated at 36°C for 3 days after which the motility was observed.

6.2.2.5 Catalase test (Slide test)

Symbiotic bacteria isolate TK1 (0.5 ml), 24 h old inoculum was placed on a clean sterile dry glass slide using a sterile loop. A drop of freshly prepared 3% Hydrogen peroxide (H_20_2) was added and mixed with the inoculum. The set up was observed for catalase reaction. To avoid false positive catalase results, a wooden stick was used instead of a metal loop.

6.2.2.6 Urease test

Urease broth and slants containing Phenol red indicator were inoculated with a loopful of 36 h old bacterial culture. The set up was incubated for 48 h to 7 days at 35°C and colour change observed.

6.3.1 Bacterial DNA extraction

Total genomic DNA was extracted from fresh bacterial cultures (24 h) grown in Nutrient broth and Nutrient agar (NA plates). The bacteria suspension (1 ml) was pippeted into Eppendorf tubes and centrifuged for 5 min at 12000 rpm after which the supernatant was decanted to get bacterial cells. The TE buffer (Tris 1.0m and EDTA 0.5m), 500 µl was added and mixed thoroughly by gentle shaking the mixture. Equal amounts 500 µl of lysis buffer were added and sample shaken gently (inverting the Eppendorfs). The Eppendorf with sample mixtures were incubated in a water bath at 37 °C for 30 min. The Phenol Chloroform Isoamyl (25:24:1 w/v) alcohol 500 µl was added to the tubes to precipitate the DNA and shaken gently (white precipitate). This was centrifuged for 10 min at 12000 rpm after which the upper layer was pipetted into fresh Eppendorfs discarding the rest. Absolute ethanol, 500 µl was added and the samples incubated for 30min at -20 °C for downstream processes. The samples were then centrifuged for 10 min at 12000 rpm to pellet the DNA. The supernatant was discarded, 1000 µl of wash buffer (95% alcohol) added and the sample mixture centrifuged for 5 min at 12000 rpm. Excess alcohol was discarded and the Eppendorfs with the sample inverted on blotting papers to air dry the DNA for 40min. The DNA was resuspended in 10 µl TAE buffer (242 g of Tris base Mwt 121.14 g, 700 ml of deionized water, 57.1 ml of acetic acid and 100 µl of 0.5M EDTA all at Ph 8.5) (Sambrook *et al.*, 1989; Weisburg *et al.*, 1991; Chen and Kuo, 1993. The presence of genomic DNA was determined by gel electrophoresis. The electrophoresis was run at 106 volts for 45 min. The gel image was viewed under digital

Imaging System, 2 UV trans illuminator and photo taken using Multi Doc-IT camera. The genomic DNA was sent to Biotech Inqabar South Africa for PCR and Sequencing.

6.3.2 Bacteria isolate TK1 sequence analysis

The sequences were analyzed using bioinformatics tools as follows: editing of the sequence was done using BioEdit Sequence Alignment Editor Sequence alignment was then done using MUSCLE on SeaView version 4 Alignment and Analysis program. A similarity search of the Genbank database was then done using Basic Local Alignment Search Tool (BLAST) at https://blast.ncbi.nlm.nih.gov. to identify the bacteria isolate TK1. The best BLAST hits obtained were retrieved and their sequences aligned using the SeaView alignment tool (Hall, 1999; Edgar, 2004; Gouy *et al.*, 2010).

6.3.3 Phylogenetic analysis of symbiotic bacteria isolate TK1

The retrieved 16S rDNA sequences were used in phylogenetic tree reconstructed using Neighbour-Joining method on SeaView program version 4 (Edgar, 2004; Gouy *et al.*, 2010) for automatic tree generation. Bootstrap analyses was performed with 1000 replications at 70% threshold for relatedness. The bacteria *Escharichia coli* (J01859) was used as an out group.

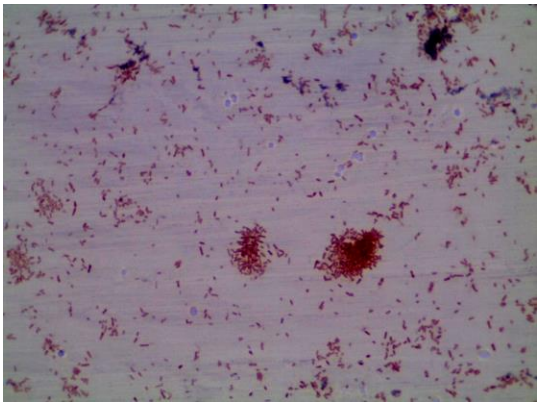
6.4 DATA ANALYSIS

The symbiotic bacteria isolate TK1 sequence and phylogeny analysis were done using BioEdit v.7.5. and SeaView v.4. Bioinformatics programmes.

6.5 RESULTS

6.5.1 Gram Staining of bacteria isolate TK1

Pink rod shaped bacterial cells were observed as the dark purple colour of primary stain (crystal violet) was not retained (Fig 6.1).



X100 **Photo:** Ngugi, CN. 2019

Figure 6.1 Gram Stain Test; Pink bacterial cells observed EPN isolate TK1 symbiont

6.5.2 Bacteria growth on differential media

Growth of cream white colonies were observed in primary and pure secondary colonies on Nutrient agar (NA) medium (Fig 6.2 A). No colony growth was observed in the control (Fig 6.2 B).

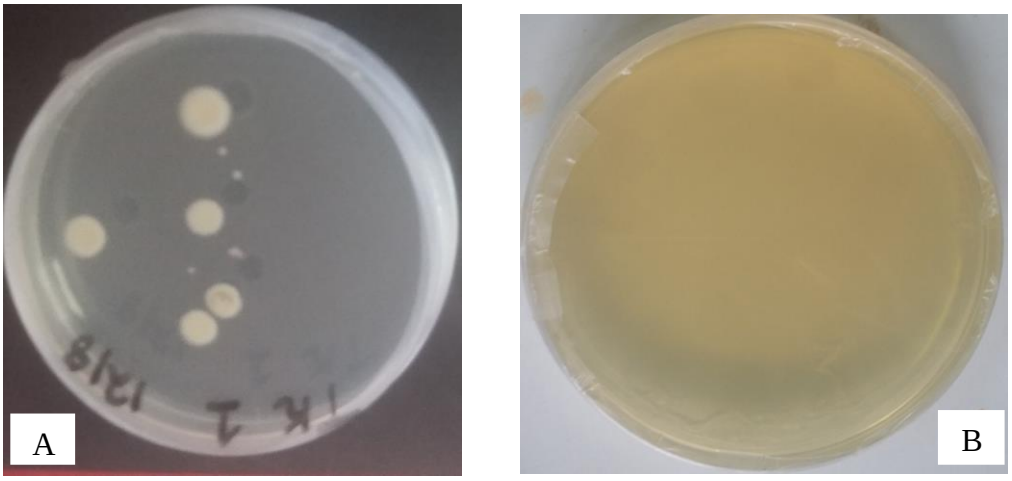


Photo: Ngugi, CN. 2019

Figure 6.2 Creamy bacterial colonies on Nutrient agar

Dark blue, convex colonies were observed on NBTA agar (Fig 6.3).

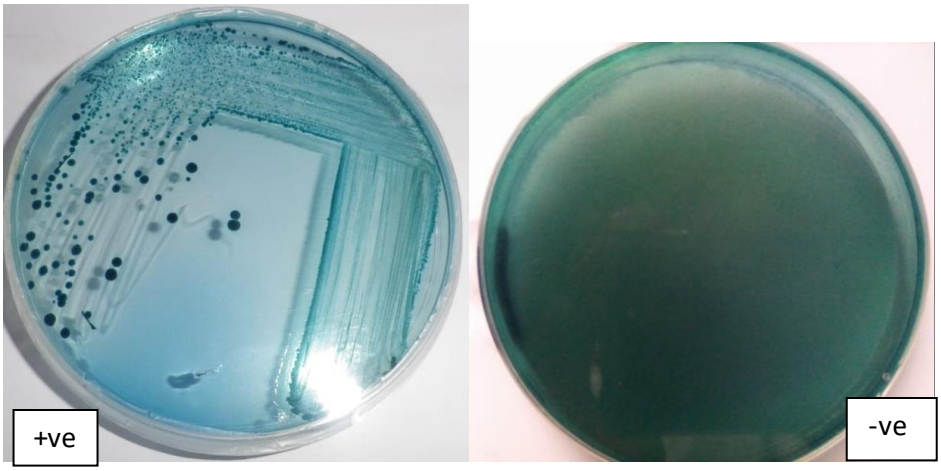


Photo: Ngugi, CN. 2019

Figure 6.3 Bacteria colonies on NBTA media
+ve, bacteria growth; -ve (control), no growth observed

Red bacterial colonies were observed on MacConkey agar plates (Fig 6.4).

No colony growth was observed in the control (uninoculated plate)

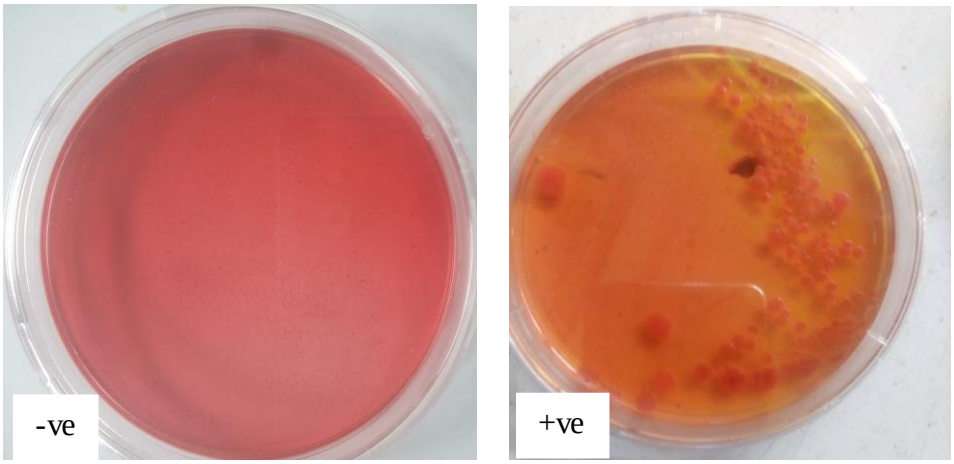


Photo: Ngugi, CN. 2019

Figure 6.4 Bacterial colonies on MacConkey media
+ve, bacteria growth; -ve (control), no growth observed

6.5.3 Glucose fermentation (Acid gas production) test

There was accumulation of gas in the Durham tubes and the medium colour changed from purple to cream-yellow. Gas bubble in the Durham tubes and change in media colour were not observed in the control experiment (uninoculated plates) after 48 h (Fig 6.5).

Gas accumulation

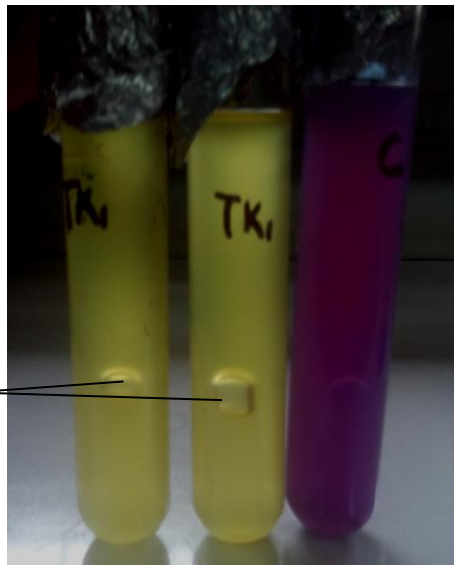


Photo: Ngugi, CN. 2019

Figure 6.5 Gas accumulation in Durham tubes and colour change from violet/purple to yellow

6.5.4 Motility test on Nutrient agar

After 48 h the media with bacteria had cloudy appearance while the control remained clear. This indicated that the bacteria isolate TK1 were motile (Fig 6.6).

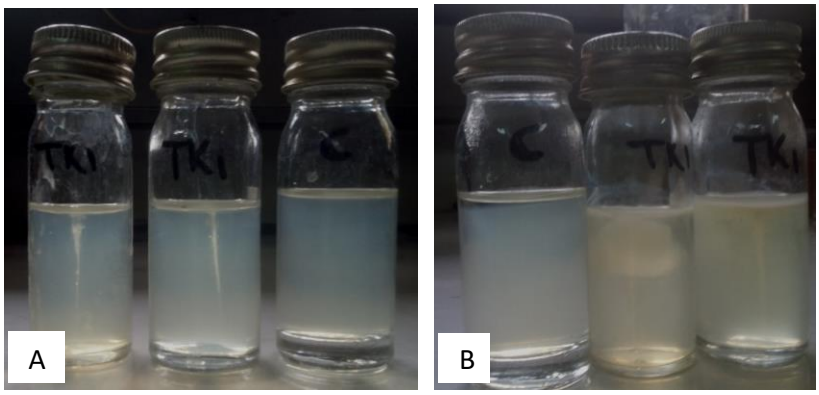


Photo: Ngugi, CN. 2019

Figure 6.6 Motility test; (A) before and (B) after 48 h

6.5.5 Catalase test

Fizzling and bubbling was not observd on the slide with bacterial isolate TK1 smear.

6.5.6 Urease test

The urease broth and agar slants with bacterial culture changed colour from cream yellow to red by the 7th day. Thus bacteria isolate TK1 was positive to urea reaction (Fig 6.7).

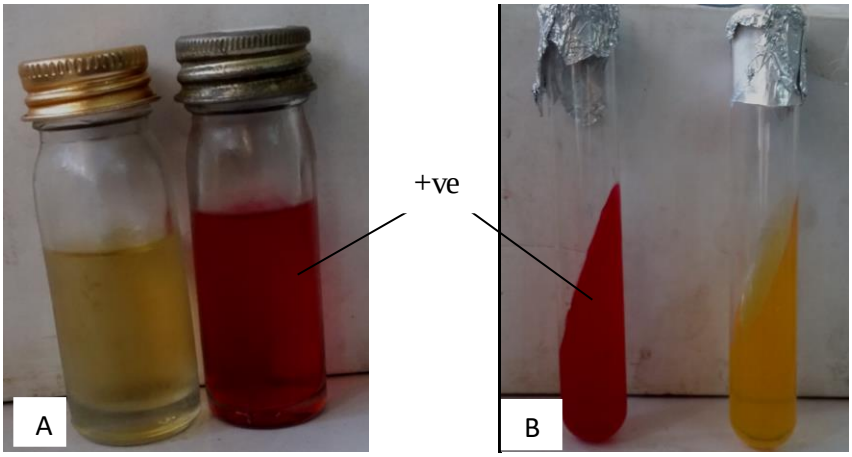


Photo: Ngugi, CN. 2019

Figure 6.7 Urease test; (A) Urease broth and (B) agar slants colour

6.6 Sequence and phylogenetic analysis

The length of 16S sequence of rDNA obtained from symbiotic bacteria TK1 was 1500 bp. (Appendix 18). The BLASTn results of 16S rDNA of the symbiotic bacteria TK1 had sequence maximum identity/similarity of between 97 and 98.93%, with *Xenorhabdus* spp retrieved from Genbank. The bacteria isolate TK1 was closely matched to *Xenorhabdus* sp. My8NJ with 98.93% similarity (Accession AB507811.1) isolated from EPN *Steinernema* sp. MY8, from Japan. Other relatives with close similarity index to the isolate were; *Xenorhabdus ishibishii* (AB243427) 98.93%, *X. ishibishii* strain GDh7 (NR117216.1) 98.79%, *X. eopokensis* DI20 (NR156925.1) 98.06%, *Xenorhabdus* sp. GD328 (GQ149085) 98.94%, *X. griffinae* ID10 (NR043643.1) 98.04%, *Xenorhabdus* sp. VN13 (FJ51800.1) 97.46%, *X. ehlersii* strain DSM (NR042327.1) 97.34%, *X. poinarii* strain NC (FJ515806) 97.27%, and *X. thuongxuanensis* (NR156924.1) with 97% identity (Appendix 19).

The selected best six BLAST hits of *Xenorhabdus* species/strains results and phylogenetic tree constructed are shown in Fig 6.8 and Appendix 19. Two major clades of bacteria

Xenorhabdus sp. were obtained from the tree. The bacteria isolate TK1 clustered in a clade sub-branch with *Xenorhabdus sp*. (AB507811.1), *X. ishibishii* (NR117216) and *X. graffinae* (NR043643) with bootstrap value of 94%. These were the most convergent *Xenorhabdus* species to bacteria isolate TK1 while *E. coli* (J01859) was below 70% bootstrap set threshold thus most divergent and unrelated among the selected bacteria species (Fig 6.8).

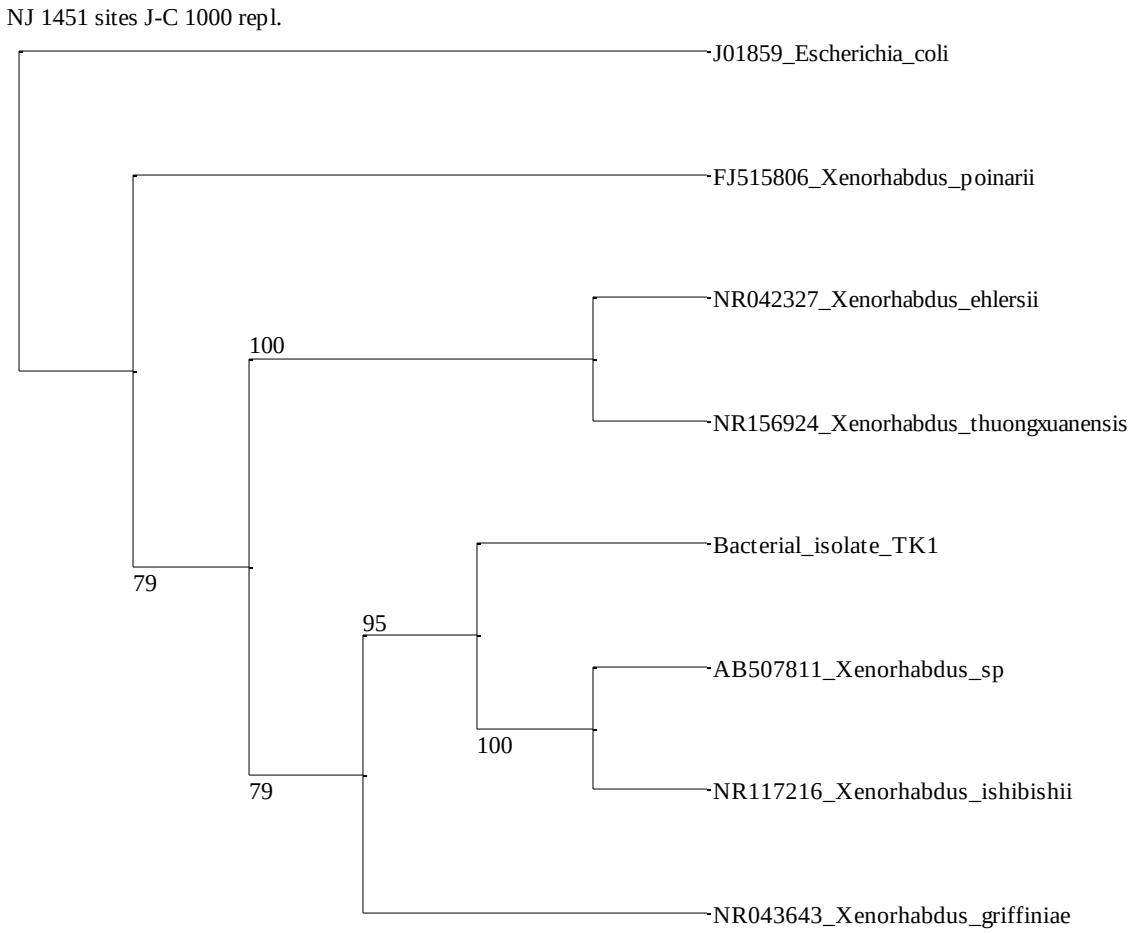


Figure 6.8 Phylogenetic relationship of bacteria isolate TK1 based on analysis of 16S rDNA sequence through Distance Method, Neighbor-Joining.

NB: Numbers at the nodes represent bootstrap through percentage

6.7 DISCUSSION

Phenotypically isolate TK1 symbiont bacterial colonies were creamy white in appearance on nutrient agar (NA) medium, raised, circular with smooth to irregular edges, shinny, and opaque in appearance. The bacterial cells were also rod shaped and the results were in conformity with Shields and Cath cart, (2011); Sugar *et al.*, (2012) and Kampfer *et al.*, (2017).

Gram stain test was used to group the EPN isolate TK1 symbiont bacteria as Gram positive or negative. Pink colouration was observed suggesting that the isolated bacteria TK1 were Gram-negative. The bacteria were unable to retain crystal violet stain (dark blue) suggesting they have a thin cell wall. It is reported that Steinernematidae EPNs harbor mutualistic bacteria, *Xenorhabdus* spp. that are Gram-negative (Kumar *et al.*, 2011; Kampfer *et al.*, 2017). The symbiotic bacteria TK1 culture indicated presence of blue colonies that left a clear zone on NBTA. Thus bacteria were able to absorb Bromothymol blue dye and reduce Triphenyltrizolium chloride (TTC) in the medium.

There was growth of red coloured bacterial TK1 colonies on MacConkey agar medium. MacConkey agar medium is selective and diferrential medium for isolation of Gram-negative rods bacteria in the family Enterobacteriaceae in which the isolated bacteria were suspected to belong. Thus growth of red coloured bacterial colonies on the medium was an indication the study bacteria was Gram - negative. This is because only Gram-negative bacteria flourish in bile salt in the MacConkey medium absorbing neutral red dye hence red colony appearance (Kumar *et al.*, 2011; Kampfer *et al.*, 2017).

Positive glucose fermentation and acid gas production were observed from isolated symbiotic bacteria TK1. This was indicated by gas accumulation in Durham tubes and change in media colour from violet/purple to yellow (Kumar *et al.*, 2011). The finding does not conform to

Thomas and Poinar, (1979) who reported glucose and other carbohydrates fermentation without acid gas production from entomopathogenic *Xenorhabdus* nematophilic bacteria.

Motility test results revealed TK1 symbiont bacteria as motile. The bacterial motility was indicated by turbidity observed extending away from the stab line of inoculation in the half strength NA medium. The medium appeared cloudy after inoculation with the bacteria. Microscopic visualisation together with media results have been used to determine motility of microorganisms in the family Enterobacteriaceae. The ability of bacteria to move is a key consideration in differentiation and classification of microorganisms (Shields and Cath cart, 2011).

The mutualistic bacteria isolate TK1 was catalase negative as no fizzling/bubbling was observed when a drop of 3% Hydrogen Peroxide (H_2O_2) was poured on bacteria smear on a slide. This suggested inability of the bacteria isolate to produce catalase enzyme that breaks down H_2O_2 into water and Oxygen. Negative catalase test results are characteristic feature of EPNs symbiotic bacteria *Xenorhabdus* and *Photorhabdus* spp. (Kumar *et al.*, 2011; Kampfer *et al.*, 2017).

Bacteria activity on urease media was positive, seven days after inoculation. The urease media, changed from yellow to red colour. This suggested that the bacteria from EPN isolate TK1 have the ability to breakdown urea into ammonia and carbon dioxide to form ammonium carbonate (alkaline) which turns indicator Phenol red bright pink from original orange yellow colour. Analysis of presence and role of ureases in EPNs in symbiont bacteria suggests that they contribute to bacterial pathogenic properties (Kumar *et al.*, 2011; Salvadori *et al.*, 2012; Lechowicz *et al.*, 2016). The results contradict with Kampfer *et al.*, (2017), who reported negative urease activity EPN symbiotic bacteria *Xenorhabdus thuongxuanensis* sp.Nov. and *Xenorhabdus eapokensis* sp.Nov. 6.7.

Molecular identification revealed the isolated symbiotic bacteria isolate TK1 was *Xenorhabdus* species. This is in line with the sequence BLASTn and phylogenetic results where all the related bacteria species were *Xenorhabdus* species. The closest relative *Xenorhabdus* sp. My8NJ (Accession AB507811.1), with 98.93% similarity to bacteria isolate TK1 and which had been isolated from EPN *Steinernema* sp. MY8, from Japan. The selected bacteria species retrieved from Genbank showed geographical relatedness in that most of them had been isolated from EPNs *Steinernema* sp. from Asian (China, Japan, Indonesia) in origin. Molecular results were also supported by the fact that the host for the bacteria isolate TK1 was found to be a *Steinernema* species of EPN based on morphological and molecular identification.

6.8 CONCLUSION AND RECOMMENDATION

From this study molecular identification confirmed the EPN isolate TK1 from which the bacteria symbiont TK1 was isolated was *Steinernema* genus of EPN. Based on biochemical and molecular analysis it is concluded that bacteria isolate TK1 is a *Xenorhabdus* species with closest relative being *Xenorhabdus* sp. My8NJ. Thus the bacteria associated with EPN isolate TK1 is *Xenorhabdus* sp. From the study it is recommended further identification and classification of this bacteria to species level to be conducted.

CHAPTER SEVEN

LARVICIDAL ACTIVITY OF BACTERIA ISOLATE TK1 AND ISOLATION OF IT'S ASSOCIATED CRUDE PROTEIN

7.1 INTRODUCTION

Agriculture contributes 30% of the gross domestic product (GDP) and 75% of employment in Kenya. Regardless of agriculture being the main driver of Kenya's economy, the country was ranked 86th out of 113 countries based on food security (PSC, 2018). Exotic vegetables accounts for 32% of horticultural production with tomato contributing 20.8% of total value. (HCD, 2016; PSC, 2018). The country is ranked 6th in Africa in terms of tomato production. Tomato growing is constrained by insect pests among them Tomato leafminer (*Tuta absoluta*). The pest is mainly managed using chemical pesticides that are associated with health and environmental safety concerns. In addition, incidences of pest resistance to chemical have been reported in most parts of the world (Haddi *et al.*, 2012; Guedes *et al.*, 2019; Bala *et al.*, 2019; Ochola *et al.*, 2019). Use of integrated pest management (IPM) strategies that include entomopathogenic nematodes (EPNs) is an alternative option for tomato pest management and sustainable yield.

The EPNs are insect parasitic nematodes used as biological pest control agents whose efficacy has been evaluated on a range of insect species. These nematodes in the families Steinernematidae and Heterorhabditae harbor symbiotic bacteria in the genus *Xenorhabdus* and *Photorhabdus* respectively (Poinar and Grewal, 2012; Yooyangket *et al.*, 2018; Salgado-Morales *et al.*, 2019). The bacterial symbiont is delivered to the homocoel of host insect pest by the EPNs on penetrating the pest through natural openings. Reproduction and pathogenicity of EPNs is dependent on nematode bacteria association. The symbiotic bacteria produce toxins or secondary metabolites that induces immunosuppression and pervasion of the haemolymph thus killing the host insect in 24 to 48 h (Aiswarya *et al.*, 2017). Nutrients

and antimicrobial agents for sterile condition necessary for reproduction and growth of EPNs are also provided by the bacteria. These bacteria symbiont have been isolated, identified and their larvicidal activity/pathogenicity assessed on economically important agricultural and medical pests (Stock *et al.*, 2017). Secondary metabolites/insecticidal proteins from the bacteria contain bioactive compounds that are antiparasitic, antifungal, antimicrobial and lethal to insects (Vanitha *et al.*, 2010; Yooyangket *et al.*, 2018). Therefore, the aim of the study was to evaluate the larvicidal effect of symbiotic bacteria isolate TK1 against *T. absoluta* second stage larvae under laboratory conditions and the isolation of crude protein from the bacteria.

7.2 MATERIALS AND METHODS

7.2.1 Larvicidal effect of symbiotic bacteria isolate TK1 against Tomato leafminer *Tuta absoluta* 2nd stage larvae

The bacterium isolate TK1 for this study was isolated from EPN isolate TK1 infective juveniles (IJs) according to Akhurst, (1980). From EPNs stock suspension, 50 µl of IJs were obtained and placed in petri dish. The IJs were surface sterilized in 1% Sodium hypochlorite for 15 min, rinsed twice in distilled water and lastly in Ringers' solution. The IJs were picked and crushed manually using a micropestle. A loopfull of the mercerated material was inoculated in Luria-Bertani (LB) broth (10 g Bacto-Tryptone, 5 g Bacto-Yeast extract and 10 g NaCl in 1lt of distilled water) and plates with NBTA media. Ten replicates were prepared for each media and incubated for 48 h at 35°C.

In addition, bacteria isolate TK1 was obtained from haemolymph of *Galleria mellonella* larvae infected with EPN isolate TK1 according to Woodring and Kaya, (1988). Four to five days after infection tan brown, odourless and firm cadavers were selected, sterilized for 5 min in 70% alcohol, flamed and allowed to dry in a laminar airflow cabinet for 2 min. Using sterile forceps and scalpel a third segment from the head was cut and the haemolymph

streaked onto nutrient agar and NBTA plates using sterile wire loop. The plates were sealed and incubated for 48 h at 35°C for bacteria growth. Single bacteria colonies were then selected and streaked onto new plates and subcultured until colonies of same size and morphology were obtained. Bacteria culture was also established in LB broth. This was then incubated for 48 h in an ENVIRON-SHAKER 3597-1 rotary incubator (Lab-Line Instruments Inc., Kerala, India) at 35°C with vigorous shaking at 150 rpm (Morgan *et al.*, 2001). For short term storage (three-four months) the bacterium was maintained on nutrient agar plates at 28°C and liquid broth at 4°C.

The isolated bacteria were used to determine pathogenicity against 2nd instar larvae of *T. absoluta*. The *T. absoluta* larvae were obtained from culture maintained on tomato crop established in a screen house at KALRO-Kandara. A modified protocol by Yooyangket *et al.*, (2018) was used. A single colony of bacteria isolate TK1 was inoculated in 3 ml of LB broth and incubated at 30°C for 48 h. The bacteria broth culture was centrifuged at 150 rpm and supernatant discarded. The bacterial pellet was resuspended in 1 ml sterile distilled water and used to evaluate larvicidal effect of the bacteria TK1 against the 2nd stage larvae of *T. absoluta*.

The experiment was comprised of five treatments with 5 replicates; control (distilled water), 25, 50, 75, and 100% bacterial concentrations. Each treatment comprised of 5 larvae individually placed in a petri dish lined with a filter paper thus a total of 25 larvae were used. In each treatment 1 ml of bacterial cell suspension was dispensed in each Petri dish. The experiment was left for 24 h, after which *T. absoluta* larval mortality was observed. Data on total number of *T. absoluta* cadavers was recorded at 24 h interval (24, 48, 72, 96 and 120 h) of exposure.

To confirm *T. absoluta* larval mortality by the bacteria, 3 tan brown, odourless and firm *T. absoluta* cadavers were randomly picked the 4th day from each treatment. The cadavers were

surface sterilized in 75% ethanol for 1 min and air dried on a filter paper. The cadavers were macerated using sterile scalpel and the haemolymph streaked on NBTA nutrient agar and culture plates incubated for 48 h at 30 °C. Growth of blue colonies indicated symbiotic bacteria isolate TK1 as the cause of death of *T. absoluta* larvae.

7.2.2 Extraction and analysis of crude protein from bacteria isolate TK1

The protein was extracted and analysed according to modified Laemmeli SDS-PAGE protocol (Fanglian, 2011). This required use of 5% acrylamide stacking gel and 7% acrylamide separation gel. The stacking gel 3 ml (2 ml double distilled water, 1.7 ml of 30% acrylamide, 25 µl of 10% Sodium dodecyl Sulfate (SDS), 25 µl of Sulphate polyacrylamide Ammoniun par Sulphate, 10 µl of TEMED and mixture swirled) was dispensed in gel caster and a small layer of ethanol or acetone added later. Set up was left for 10-15 min to set. The acrylamide stacking gel (2 ml double distilled water, 0.506 ml of 30% acrylamide, 0.1 ml of tris HCL ph 6.8, 15 µl of SDS, 15 µl Ammonium per Sulphate and 10 µl of TEMED-Tetramethylethylenediamine (by increasing from 4 to 10 µl to enhance solidification) was later dispensed onto the separation gel.

From 48 h bacteria LB broth, 1.5 ml of culture was pippeted and centrifuged at 8000 rpm for 5 min. The supernatant was discarded and the pellet resuspended in loading dye. The suspension was denatured by heating in a Thymocycler at 100 °C for 15-20 min and transferred to PCR tubes. The sample was diluted with equal volume of SDS.

The crude extract (8 µl) samples were loaded into the gel caster wells (Hoefer Dual Gel Caster, Amershan, UK). The gel electrophoresis was run first at 35 volts for 45 min to allow for stratification of proteins according to their relative charge. Protein Standard marker of molecular weight (Mwt) 250 kDa (Amersham Biosciences, UK), was also included in this gel. The voltage was increased to 100°C for 1 h until the dye marker was at the bottom gel end moved to lower end of the gel.

The resulting crude protein profiles was visualized on Coomassie brilliant blue (R-250) stained native-PAGE gels. The gel was fixed first in 10% glacial acetic acid (HOAC), 50% Methanol (MeOH), and 40% H_2O for 25-30 min. The gel was stained with 0.25% Coomassie (100 ml glacial acetic acid in 450 ml of double distilled water and 3 g Coomassie dye in 450 ml Methanol all filtered) for 2-4 h to get even colour. Destaining of the gel in 5% MeOH, 7% HOAC, 88% H_2O was done for 12 h until the background was clear. The protein bands were viewed under UV lamp and molecular weight assessed against the marker. Gel images were also taken and the gel was maintained in 7% HOAC.

7.3 DATA ANALYSIS

Data on mortality against time and concentration was subjected to Statistical analysis, Analysis of variance (ANOVA) using Genstat, 15th edition, Statistical software. The means were separated using Fisher's protected least significant difference test at 5% significance level.

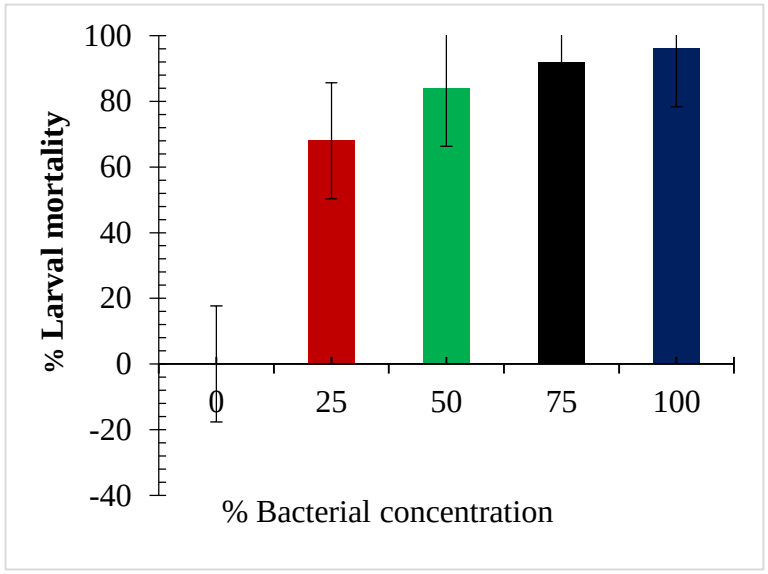
7.4 RESULTS

7.4.1 Larvicidal effect of bacteria isolate TK1 against the 2nd stage larvae of *Tuta absoluta* under laboratory condition

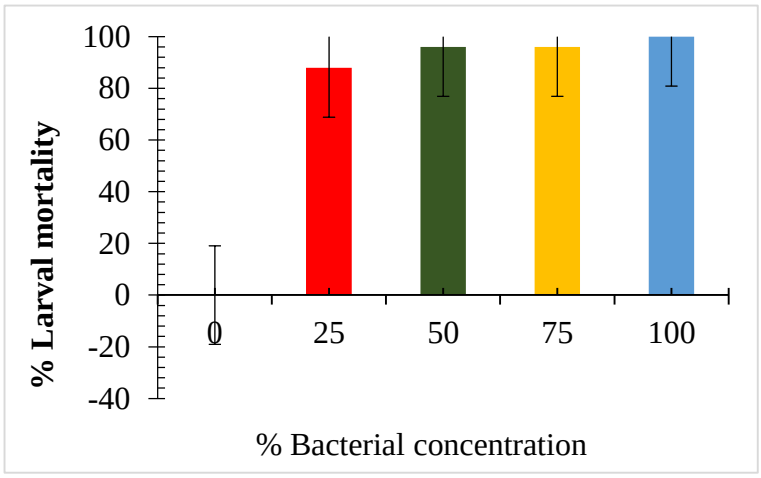
Larval mortality of *Tuta absoluta* was observed only in the bacteria treatments over 48 h of exposure. In the first 24 h of exposure, there was no larval mortality recorded in the control experiment. The larval mean mortality of 68±4.9, 84±4.0, 92±4.9, and 96±4.0 %, respectively, was observed in bacteria concentrations 25, 50, 75 and 100% (Fig 7.1 A). There was significant difference ($P<0.05$) between control and all the other treatments. A significant difference ($P<0.05$) between bacteria concentration 25, 50, 75 and 100 % was observed (Appendix 19 and 20).

At 48 h exposure time mean mortality of 0±0.0, 88±8.0, 96±4.0, 96±4.0 and 100±0.0% was recorded in the control and bacteria concentrations 25, 50, 75 and 100% respectively (Fig 7.1

B). There was significant difference (P<0.05) between the control and all the treatments but there was no significant difference among the treatments with bacteria (Appendix 20).



A



B

Figure 7.1 Larvicidal effect of bacteria isolate TK1 on 2nd instar larvae of *Tuta absoluta* (A) at 24 h and (B) 48 h exposure

7.4.2 Comparison between exposure time and mortality of 2nd instar larvae of *Tuta absoluta* due to bacteria isolate TK1

There was larval mortality in 24 and 48 h exposure time. Overall mortality at time 24 h was 68±7.394 % while at time 48 h it was 76±8.00 %. There was no significant difference (P >0.05 between larval mortality at 24 and 48th h of exposure TK1 (Fig 7.2; Appendix 21).

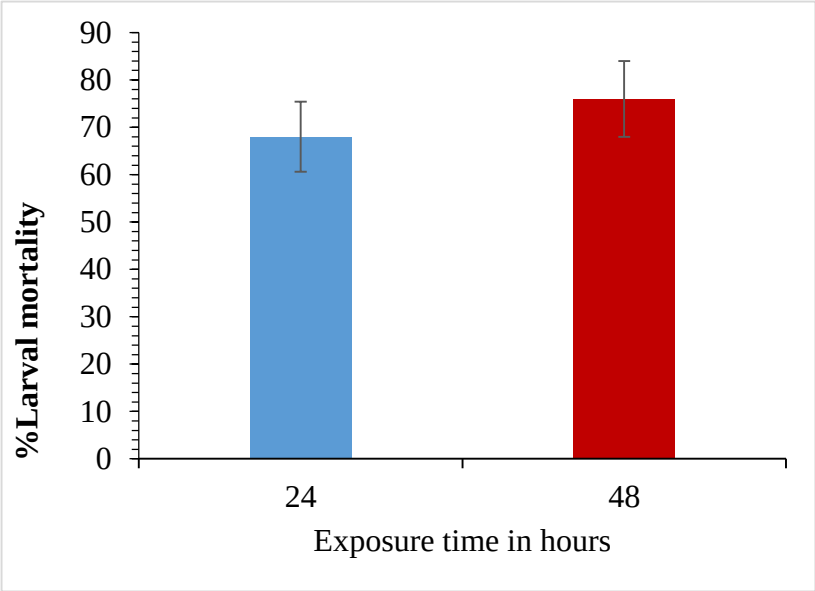


Figure 7.2 Mortality of 2nd larvae of *Tuta absoluta* at 24 and 48 h exposure period

7.4.3 Bacteria isolate TK1 crude protein SDS-PAGE analysis

Three distinct bands of crude protein from bacteria isolate TK1 SDS-PAGE profile were observed under UV lamp. The Sa and Sb were the protein samples and developed similar bands. The molecular weight for the two samples were 100 kDa to 240 kDa (Fig 7.3).

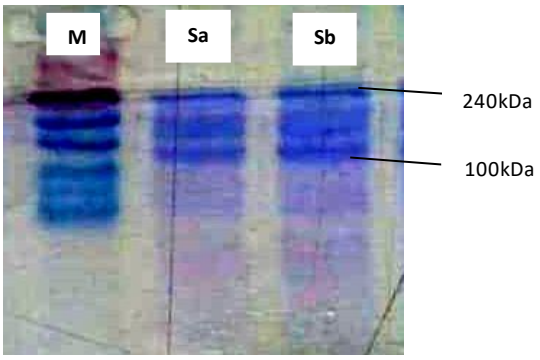


Photo**:** Ngugi, CN, 2019

Figure 7.3 Bacteria isolate TK1crude protein bands on SDS-PAGE Gel image
Sa and Sb, Protein samples from bacteria TK1; M, Standard protein marker of 250 kDa

7.5 DISCUSSION

Results from this study showed that bacteria isolate TK1 was able to infect and kill *Tuta absoluta* larvae, meaning that *T. absoluta* larvae were susceptible to bacteria isolate TK1. It is suggested that, bacteria isolate TK1 were able to overcome *T. absoluta* immune resistance leading to death of the larvae. It was observed that even outside EPN vector, bacteria isolate TK1 was still lethal to *T. absoluta* larvae. There is a possibility that the bacterial cells entered the larvae haemocoel through the same natural openings (anus, mouth, spiracles), which have been found to be entry points for EPN. Similar findings have been reported by Shan *et al.* (2019). This is the first scientific research report on EPNs symbiotic bacteria larvicidal effect against *T. absoluta* larvae in Kenya. However, some previous works have reported larvicidal activity of EPNs symbiotic bacterial cell suspensions against insect larvae of Diamond back moth (*Plutella xylostella*), Greater wax moth (*Galleria mellonella*) and mosquito (*Aedes aegypti* and *Aedes albopictus*) (Vanitha *et al.*, 2010; Vitta *et al.*, 2018; Yooyangket *et al.*, 2018; Salgado-Morales *et al.*, 2019).

When *T. absoluta* larvae was exposed to different bacterial cell concentrations overtime, it resulted to varied percentage larval mortality. The lower bacteria cell concentrations caused lower larval mortality while higher mortality was observed from higher cell concentration in the first 24 h of exposure. This indicated possibility of high pest knock down with increase in

bacterial cell concentration just like conventional insecticide. The results agree with Kalia *et al.*, (2018), who reported mortality of Tobacco cutworm (*Spodoptera litura*) neonates after 96 h of exposure to *Photorhabdus luminescens* bacteria following feeding bioassay. There was no significant difference in % *T. absoluta* larval mortality at exposure time 24 and 48 h. This result suggested that pathogenicity of bacteria isolate TK1 was not dependent on exposure period but mainly by bacterial cell concentrations and protein toxins in the bacterial cells.

Analysis of crude protein from the study bacteria revealed presence of 3 main protein complexes as depicted by bands in the gel electrophoresis image. The bacteria isolate TK1 protein complexes had moleculer weight (Mwt) ranging between 100 and 240 kDa. Protein complexes of different molecular weight from EPNs symbiotic bacteria have been isolated, purified, identified and their toxicity tested on insects by other scientists. The toxic compounds (Tc) have been found to be lethal against varous insect pests. According to Sheet *et al.*, (2011), three classes (A, B and C) of toxin complexes with Mwt 280, 170 and 110 kDa respectively were isolated from EPN bacteria *Xenorhabdus nematophila*. In addition, Wang *et al.*, (2012) isolated bacterial protein (Txc 40) from EPN bacteria *Xenorhabdus nematophilia* and was toxic to *P. xylostella* larvae.

7.6 CONCLUSION AND RECOMMENDATION

Bacteria isolate TK1 was found to be pathogenic to *Tuta absoluta* larvae with 68% to 100% larval mortality being attained in the first 24 h at different concentrations. This meant that the bacteria isolate TK1 were able to enter host insect body outside its EPN vector. The *T. absoluta* larvae are highly susceptible to the bacteria bacteria isolate TK1 suggesting ability to suppress host resistance. The bacteria isolate TK1 has the potential to be used as biological control agent for *T. absoluta*. The isolate harbours 3 major protein complexes as depicted by bands in the gel image.

Further investigation is required to ascertain bacterial cells motility features and route of entry into the *T. absoluta* body. Evaluation of bacteria TK1 cells viability outside the nematode vector is necessary. Screen house and field trials should be conducted to ascertain larvicidal effect of bacteria isolate TK1 on *T. absoluta* larvae on tomato plants. Purification and biochemical analysis of bacterial crude proteins is recommended to determine the insecticidal compounds.

CHAPTER EIGHT

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

8.1 GENERAL DISCUSSION

The study results revealed that all the selected indigenous entomopathogenic nematodes (EPNs) were infective against *Tuta absoluta* larvae as they caused larval mortality and infective juveniles recovered. This suggests that the EPNs enterered, reproduced and killed *T. absoluta* larvae. The *T. absoluta* was susceptible to the five EPNs but degree of infection was dependent on the specific EPN isolate. However, isolate TK1 was found to be the most infective on *T. absoluta* larvae. Isolate TK1 caused the highest *T. absoluta* mortality within the least time of 24 h and at the lowest EPN concentration of 100 infective juveniles (IJs)/ml of distilled water. In addition, isolate 97 was the least infective on *T. absoluta* larvae. This finding may be attributed to EPNs host preference, finding and foraging behavior. The results are in conformity with Gozel *et al.*, 2015, who reported infection of EPN on *T. absoluta* larvae, with *Steinernema feltiae* being the most infective where as *S. affinae* the least.

Morphological study demonstrated EPN isolate TK1 as *Steinernema* sp. in the *feltiae-glaseri* group of EPNs. The isolate had close features with the selected known EPN species which was supported by molecular analysis of ITS region of rDNA. Sequence analysis, for similarity implied that EPN isolate TK1 was closely related (83% to 92%,) to Eastern Africa and Asia *Steinernema* spp. EPNs. These EPNs included, from Kenya (AY230186.1), Sri Lanka, (AY230184.1), Tanzania (KT358812.1), *S. karii* from Kenya (AY230173.1), and *S. ethiopense* (JN651414.1), from Ethiopia among others. These regions are characterized by warm tropical climate. Climate is documented as a key factor influencing distribution and occurrence of EPN species. Biogeographical distribution of the EPN species is influenced by host distribution, behavioral and physiological adaptations, and soil type (Campos-Herrera *et al.*, 2012; Birhan *et al.*, 2017). Evolutionary relationship based on phylogeny analysis of

EPN isolate TK1 sequence was observed as clustered together with close EPN *Steinernema* sp. and distance from the divergent EPN based on Bootsrap values.

The morphological and biochemical study of symbiotic bacteria from EPN isolate TK1 established presence of *Xenorhabdus* species of bacteria. The isoltae bacteria TK1 colonies were circular with smooth to irregular edges, shinny, and opaque in appearance. The bacterial cells were also rod shaped, gram negative, motile, urease positive, catalase negative, blue on NBTA and positive for glucose fermentation test and acid gas production. The findings were in line with Shields and Cath cart, (2011) and Kampfer *et al*., (2017) who reported positive for glucose fermentation test and acid gas production. However, the positive bacteria urease test results disagree with Kampfer *et al*., (2017), who reported negative urease test from *Xenorhabdus* sp. bacteria.

Molecular identification revealed the isolated symbiotic bacteria TK1 was *Xenorhabdus* species. This is in line with 16S region of rDNA sequence and phylogenetic analysis results where all the related bacteria species were *Xenorhabdus* species. The closest relative of bacteria isolate TK1 was *Xenorhabdus* sp. My8NJ (Accession AB507811.1), with 98.93% similarity and which had been isolated from EPN *Steinernema* sp. MY8, from Japan. The bacteria isolate TK1 molecular results were also supported by the fact that the host was a *Steinernema* sp. of EPN, based on morphological and molecular identification. The *Xenorhabdus* sp. My8NJ (Accession AB507811.1) and study bacteria isolate TK1 are similar in that their host EPNs are not identified to species level and suspected to be new species. In addition, the two bacteria nematode hosts are from tropics hence Afro-Asia in origin indicative of evolutionary relationship. The bacteria isolate TK1 close relatives from Genbank were from Asia; Japan (AB507811.1 *Xenorhabdus* sp. My8NJ, AB243427 *X. ishibishii*); China (NR117216.1 *X. ishibishii* strain GDh7, GQ149085 *Xenorhabdus* sp. GD328 and NR042327.1 *X. ehlersii* strain DSM) and *X. griffinae* ID10 (NR043643.1) from

Indonesia, an indication of evolutionary relationship. This finding confirms association between EPN *Steinernem*a spp. and symbiotic bacteria *Xenorhabdus* spp. (Ferreira *et al.*, 2014; Lalramnghaki *et al.*, 2017; Yooyangket *et al.*, 2018).

The isolated bacteria TK1 was pathogenetic against 2nd larval stage of *T. absoluta*. The bacteria were able to infect the larvae outside the vector EPN isolate TK1. It is suspected that the bacteria gained entry into the *T. absoluta* larvae through natural body orifices reaching the homeocoel where they multiplied causing septicaemia hence death of the larvae. The bacteria were recovered from *T. absoluta* cadaver and which confirmed its larvicidal activity. Similar findings have been reported on insect larvae of Diamond back moth, (*Plutella xylostella*), *G. mellonella*, mosquito (*Aedes aegypti* and *Aedes albopictus*), (Vanitha *et al.*, 2010; Vitta *et al.*, 2018; Yooyangket *et al.*, 2018; Salgado-Morales *et al.*, 2019).

The study further demonstrated that symbiotic bacteria TK1 contains a range of proteins. This was indicated by presence of three bands of different molecular weight in the SDS-PAGE gel electrophoresis. Entomopathogenic nematodes symbiotic bacteria are known to produce antibiotics and enzymes in addition to protein toxins that makes them lethal to insect pests. This agrees with previous studies on pathogenicity of EPN symbotic bacteria (Li *et al.*, 2009; Sheet *et al.*, 2011; Ruiu *et al.*, 2013).

8.2 CONCLUSIONS

From the study it was concluded that the entomopathogenic nematode (EPN) isolate TK1 was a *Steinernema* species in the feltiae-glaseri group of EPNs and its symbiotic bacteria isolate TK1 is *Xenorhabdus* spp. Both the EPN isolate TK1 and symbiotic bacteria isolate TK1 (outside its EPN vector) were pathogenic against *Tuta absoluta*. The study enriched diversity of indigenous EPNs and associated bacteria, providing information on potential biological control for the management of *T. absoluta*.

8.3 RECOMMENDATIONS

8.3.1 Recommendations from the study

- The most pathogenic entomopathogenic nematode (EPN) isolate TK1 should be packaged and availed to tomato farmers for management of *Tuta absoluta*
- Symbiotic bacteria isolate TK1 should be cultured in large numbers and used in *T. absoluta* management
- Crude protein from bacteria isolate TK1 is also recommended for use in integrated pest management (IPM) option for *T. absoluta*

8.3.2 Recommendations for future work

- Screening of infectivity of the entomopathogenic nematode isolates 75, S86, R52, 97 and TK1 on *Tuta absoluta* eggs and pupal stages
- Characterisation of entomopathogenic nematodes (EPNs) isolates 75, S86, R52 and 97 at least to genus and TK1 to species level to establish their identity
- Conduct screen house and field trials to validate the laboratory results on infectivity of EPN isolate TK1 against *T. absoluta* larvae
- Further morphological investigations using Scanning Electron Microscope (SEM) to ascertain and identify EPN isolate TK1 to species level as it was suspected to be a new species
- The EPN isolate TK1 should be tested for effectiveness on other vegetable pests of economic importance especially other tomato pests
- Further investigation is required to ascertain isolate bacterial TK1 cells motility features aiding penenetration and entry route into the body of *T. absoluta*
- Evaluation of bacteria cells viability outside the EPN vector is required

- Screen house and field trials should be conducted to validate larvicidal effect of the bacteria TK1 on *T. absoluta* larvae on tomato pest
- Purification and biochemical analysis of bacterial crude proteins is recommended to identify and quantify toxic compound present

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APPENDICES

Appendix 1 Effect of entomopathogenic nematode isolates concentrations (100) and time on mortality of *Tuta absoluta* 2nd instar larvae

EPN isolates	Time of exposure				
	24	48	72	96	120
75	20±11.0a	76±7.5a	84±4.0a	96±4.0a	96±4.0a
97	20±12.6a	48±8.0a	60±12.6a	76±4.0a	96±4.0a
R52	40±11.0a	76±9.8a	88±4.9a	100±0.0a	100±0.0a
S86	40±6.3a	84±7.5a	96±4.0a	100±0.0a	100±0.0a
TK1	60±8.9a	92±4.9a	100±0.0a	100±0.0a	100±0.0a
P-value	>0.001	>0.001	>0.001	>0.001	>0.001

Appendix 2 Effect of entomopathogenic nematode isolates concentrations (150) and time on mortality of *Tuta absoluta* 2nd instar larvae

EPN isolates	Time of exposure				
	24	48	72	96	120
75	56±17.2 hi	72±15.0defgh	84±7.5 abcde	96±4.0ab	100±0.0 a
97	12±4.9k	36±4.0 j	68±12.0efghi	100±0.0 a	100±0.0 a
R52	52±4.9 ij	76±7.5cdefg	92±4.9abc	100±0.0a	100±0.0 a
S86	60±6.3ghi	64±7.5fghi	88±4.9abcd	100±0.0a	100±0.0a
TK1	80±6.3bcdef	96±4.0ab	100±0.0a	100±0.0a	100±0.0 a
P-value	<0.001	<0.001	<0.001	<0.001	>0.001

Appendix 3 Effect of entomopathogenic nematode isolate concentrations (200) and time on mortality of *Tuta absoluta* 2nd instar larvae

EPN isolates	Time of exposure				
	24	48	72	96	120
75	32±10.2 h	68±10.2 def	84±7.5abcd	96±4.0ab	100 ±0.0a
97	12±8.0 I	44±7.5gh	68 def	92abc	100±0.0 a
R52	48±4.9 gh	88±8.0abc	100±0.0 a	100±0.0a	100±0.0a
S86	52±13.6fg	76±7.5cde	96±4.0ab	100±0.0a	100±0.0a
TK1	60±6.3 efg	80±6.3bcd	92±4.9abc	100±0.0a	100±0.0a
P-value	< 0.001	< 0.001	< 0.001	< 0.001	> 0.001

Appendix 4 Effect of entomopathogenic nematode isolate concentrations (250) and time on mortality of *Tuta absoluta* 2nd instar larvae

EPN isolates	Time of exposure				
	24	48	72	96	120
75	40±8.9f	64±4.0cd	92±4.9ab	100±0.0 a	100±0.0 a
97	4±4.0g	40±8.9f	76±9.8bc	100±0.0 a	100±0.0a
R52	44±7.5ef	84±7.5ab	96±4.0a	100±0.0 a	100±0.0a
S86	60±11.0cde	84±7.5ab	92±4.9ab	100±0.0a	100±0.0a
TK1	56±7.5def	76±9.8bc	88±8.0 ab	100±0.0a	100±0.0a
P-value	< 0.001	< 0.001	< 0.001	>0.001	> 0.001

Appendix 5 Comparison between effect of entomopathogenic nematode isolate concentration (100) on 2nd and 3rd larval stage of *Tuta absoluta*

T. absoluta	EPN isolates				
	75	97	R52	S86	TK1
3rd stage	80±6.0a	59.2±8.37a	86.4±4.43a	88.8±3.48a	91.2±3.67a
2nd stage	74.4±6.38 a	60±6.43a	80.8±5.35a	84±5.03a	90.4±3.67a
P-value	>0.001				

Appendix 6 Comparison between effect of entomopathogenic nematode isolate concentration (150) on on 2nd and 3rd larval stage of *Tuta absoluta*

T. absoluta	E PN isolates				
	75	97	R52	S86	TK1
3rd stage	91.2±3.84a	60±8.87a	85.6±3.75a	84.8±4.21a	92.8±3.24a
2nd stage	81.6±5.53a	63.2±7.54a	84±4.16a	82.4±4.05a	95.2±2.09a
P-value	>0.001				

Appendix 7 Comparison between the effect of entomopathogenic nematode isolate concentration (200) on on 2nd and 3rd larval stage of *Tuta absoluta*

T. absoluta	EPN isolates				
	75	97	R52	S86	TK1
3rd stage	87.2±4.14a	64±8.17a	92±3.65a	88.8±4.63a	84±4.16a
2nd stage	79.2±5.23a	64±8.0a	84.8±4.8a	87.2±3.98a	84±4.16a
P-value	>0.001				

Appendix 8 Comparison between the effect of entomopathogenic nematode isolates concentration (250) on on 2nd and 3rd larval stage of *Tuta absoluta*

T. absoluta	EPN isolates				
	75	97	R52	S86	TK1
3rd stage	81.6±5.15a	61.6±8.40a	88.8±4.33a	88±4.16a	83.2±3.77a
2nd stage	76±5.89a	63.2±7.35a	87.2±4.45a	84.8±4.8a	86.4±3.60a
P-value	>0.001				

Appendix 9 Interation between entomopathogenic nematode isolates and time on larval mortality (24 h)

Source of variation	d.f.	s.s.	m.s	v.r.	F pr.	
Rep	4	87.68	21.92	0.34		
Isolate	4	13105.28	3276.32	50.18	<.001	***
stage	1	72	72	1.1	0.301	
Isolate.stage	4	553.6	138.4	2.12	0.098	
Residual	36	2350.72	65.3			
Total	49	16169.28				

Appendix 10 Interaction between entomopathogenic nematode isolates and time on larval mortality (48 h)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep	4	426.88	106.72	2.08	
Isolate	4	9582.08	2395.52	46.74	<.001
stage	1	115.52	115.52	2.25	0.142
Isolate.stage	4	442.88	110.72	2.16	0.093
Residual	36	1845.12	51.25		
Total	49	12412.48			

Appendix 11 Interaction between entomopathogenic nematode isolates and time on larval mortality (72 h)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep	4	296.32	74.08	1.91	
Isolate	4	2853.12	713.28	18.35	<.001
stage	1	72	72	1.85	0.182
Isolate.stage	4	140.8	35.2	0.91	0.471
Residual	36	1399.68	38.88		
Total	49	4761.92			

Appendix 12 Interaction between entomopathogenic nematode isolates and time on larval mortality (96 h)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep	4	6.4	1.6	0.43	
Isolate	4	128	32	8.57	<.001
stage	1	15.68	15.68	4.2	0.048
Isolate.stage	4	43.52	10.88	2.91	0.035
Residual	36	134.4	3.733		
Total	49	328			

Appendix 13 Interaction between entomopathogenic nematode isolate and time on mortality (120 h)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep	4	1.92	0.48	0.73	
Isolate	4	1.92	0.48	0.73	0.578
stage	1	1.28	1.28	1.95	0.172
Isolate.stage	4	1.92	0.48	0.73	0.578
Residual	36	23.68	0.6578		
Total	49	30.72			

Appendix 14 Morphometrics of infective juveniles of entomopathogenic nematode isolate TK1

n	L	a	b	c	c′	MBD	EP	ES	TL	ABD	H	D%	E%	H%
1	892.52	18.15	7.75	13.66	2.64	49.18	80.51	115.14	65.34	24.76	16.55	69.92	231.65	1.85
2	736.2	18.74	6.52	12.97	2.72	39.28	85.14	112.92	56.75	20.86	18.4	75.40	282.05	2.50
3	848.46	18.61	7.41	11.86	2.81	45.6	83.31	114.48	71.51	25.41	20.09	72.77	219.02	2.37
4	976.21	18.61	9.68	20.16	2.17	52.45	78.58	100.88	48.42	22.36	24.73	77.89	305.10	2.53
5	817.19	18.31	11.04	12.46	2.77	44.62	82.97	74.01	65.6	23.7	13.45	112.11	237.78	1.65
6	986.89	17.85	9.74	15.50	2.91	55.3	95.57	101.34	63.66	21.85	24.3	94.31	282.24	2.46
7	903.35	19.16	8.19	17.13	2.29	47.15	89.01	110.29	52.74	23.07	20.01	80.71	317.29	2.22
8	762.17	16.46	7.52	17.99	2.27	46.3	62.66	101.39	42.36	18.7	23.99	61.80	278.09	3.15
9	730.51	16.74	6.79	14.49	2.45	43.65	78.19	107.57	50.42	20.54	19.34	72.69	291.55	2.65
10	844.95	16.51	7.53	15.42	2.68	51.18	79.68	112.21	54.8	20.45	15.78	71.01	273.35	1.87
11	658.6	16.90	5.90	16.34	2.08	38.98	73.11	111.58	40.3	19.4	20.49	65.52	341.06	3.11
12	829.31	17.50	7.28	15.77	2.84	47.38	79.09	113.94	52.58	18.5	21.46	69.41	282.79	2.59
13	763.22	17.08	7.04	17.12	2.42	44.68	72.02	108.37	44.58	18.4	18.76	66.46	303.72	2.46
14	869.97	19.70	7.48	20.45	2.21	44.17	82.32	116.38	42.54	19.25	22.32	70.73	363.80	2.57
15	908.28	19.55	7.88	16.80	2.11	46.46	89.97	115.23	54.06	25.63	17.28	78.08	312.88	1.90
16	841.15	16.26	7.08	15.08	2.24	51.73	83.93	118.86	55.78	24.95	19.5	70.61	282.88	2.32
17	925.97	18.06	8.45	17.02	2.39	51.28	87.93	109.62	54.42	22.81	24.95	80.21	303.76	2.69
18	711.13	16.60	6.29	14.47	2.98	42.85	78.42	113	49.15	16.5	23.97	69.40	299.96	3.37
19	862.19	17.56	12.15	18.25	1.82	49.11	80.98	70.98	47.25	25.97	24.93	114.09	322.21	2.89
20	822.55	17.46	7.97	15.81	2.17	47.12	81.17	103.25	52.04	23.96	19.08	78.62	293.24	2.32
Average	834.54	17.79	7.98	15.94	2.45	46.92	81.23	106.57	53.22	21.85	20.47	77.59	291.22	2.47
SD	87.36	1.06	1.57	2.30	0.33	4.23	7.06	12.78	8.35	2.83	3.33	14.00	34.69	0.45
Min	658.60	16.26	5.90	11.86	1.82	38.98	62.66	70.98	40.30	16.50	13.45	61.80	219.02	1.65
Max	986.89	19.70	12.15	20.45	2.98	55.30	95.57	118.86	71.51	25.97	24.95	114.09	363.80	3.37

Appendix 15 Morphometrics of male of entomopathogenic nematode isolate TK1

n	L	a	b	c	MBD	EP	ES	TL	AMD	SL	GL	D%	E%	SW%	GS%
1	2096.87	17.95	17.32	92.95	116.83	100.58	121.06	22.56	33.63	83.49	54.87	83.08	445.83	71.46	65.72
2	1882.57	16.95	17.11	113.54	111.06	98.06	110.04	16.58	33.2	73.06	45.72	89.11	591.44	65.78	62.58
3	1737.98	13.52	14.48	85.07	128.52	98.99	120.04	20.43	45.03	75.01	45.43	82.46	484.53	58.36	60.57
4	1666.64	15.54	14.24	69.73	107.26	105.03	117.06	23.9	28.32	76.84	35.68	89.72	439.46	71.64	46.43
5	1698.97	15.92	14.55	89.47	106.75	94.12	116.8	18.99	37.89	57.86	49.92	80.58	495.63	54.20	86.28
6	2081.82	12.85	13.81	125.56	162.06	130.22	150.77	16.58	27.51	82.49	42.92	86.37	785.40	50.90	52.03
7	2030.4	12.48	19.44	115.36	162.74	103.76	104.44	17.6	34.4	86.32	45.13	99.35	589.55	53.04	52.28
8	1629.99	15.70	15.83	76.63	103.85	90.26	102.96	21.27	37.39	76.87	44.64	87.67	424.35	74.02	58.07
9	1708.35	16.31	13.45	72.45	104.74	109.12	126.97	23.58	37.4	71.46	43.42	85.94	462.77	68.23	60.76
10	1727.4	17.45	15.76	121.65	99.01	92.17	109.6	14.2	30.4	77.46	42.34	84.10	649.08	78.23	54.66
11	2019.52	13.91	18.57	104.42	145.17	84.51	108.76	19.34	39.82	128.3	54.45	77.70	436.97	88.38	42.44
12	1793.81	19.45	14.38	95.98	92.23	112.8	124.72	18.69	32.66	82.57	45.83	90.44	603.53	89.53	55.50
13	1633.54	18.11	12.32	78.50	90.21	109.78	132.55	20.81	32.39	79.13	43.9	82.82	527.53	87.72	55.48
14	1958.46	18.47	13.49	99.26	106.03	140.13	145.14	19.73	28.4	90.77	37.79	96.55	710.24	85.61	41.63
15	1873.73	18.87	15.17	102.67	99.28	120.9	123.53	18.25	30.25	64.49	38.21	97.87	662.47	64.96	59.25
16	1296.62	12.43	10.76	57.17	104.31	108.58	120.54	22.68	30.17	93.23	34.48	90.08	478.75	89.38	36.98
17	1762.89	15.37	15.63	81.58	114.72	102.45	112.81	21.61	31	90.15	52.25	90.82	474.09	78.58	57.96
18	1573.96	15.30	12.87	87.44	102.84	117.05	122.33	18	25.62	87.71	52.94	95.68	650.28	85.29	60.36
19	1776.42	18.59	14.26	69.12	95.57	115.13	124.59	25.7	38.79	84.57	49.34	92.41	447.98	88.49	58.34
20	1676.66	14.45	18.01	88.38	116.07	93.83	93.1	18.97	30.41	79.17	46.43	100.78	494.62	68.21	58.65
Average	**1781.33**	**15.98**	**15.07**	**91.35**	**113.46**	**106.37**	**119.39**	**19.97**	**33.23**	**82.05**	**45.28**	**89.18**	**542.72**	**73.60**	**56.30**
SD	**195.07**	**2.22**	**2.19**	**18.60**	**20.87**	**13.77**	**13.57**	**2.84**	**4.87**	**13.94**	**5.89**	**6.49**	**105.13**	**13.01**	**10.32**
Min	**1296.62**	**12.43**	**10.76**	**57.17**	**90.21**	**84.51**	**93.10**	**14.20**	**25.62**	**57.86**	**34.48**	**77.70**	**424.35**	**50.90**	**36.98**
Max	**2096.87**	**19.45**	**19.44**	**125.56**	**162.74**	**140.13**	**150.77**	**25.70**	**45.03**	**128.30**	**54.87**	**100.78**	**785.40**	**89.53**	**86.28**

Appendix 16 ITS rDNA sequence for entomopathogenic nematode isolate TK1

TGGAAGGACTACGTGTTCCTGTGCCTGCAGACTCGAGGGGGAGGGCACTAGGCGAACGT
ATATTTGTCTCCTGATACGCAGATCGATAGCCCGAGCTATCCATTACCTGATTTGATTGG
ACGGCGTCGCTACGGTTCTAAGCGTCGATTCCGGTCGCAAACGGCTTTGAATGGTTCCTA
TAGATGTCTGGAGCAGCTGTATGAGCGTGGCTGTGGTGATGGACATAGACGTTGCTTGAG
CGTGCTTCTGTTTCTAGCGCTCTGACTATAGCGACGAAGAATTAAAGAGGTCAGTCGGAG
ACCCGCCGTTCTTATAAAACTACTTTTAACATTTTTCACGATGCTGTGCCATGCGAATGGT
GCAAAAAGACTATTATCAAGTCTTATCGGTGGATCACTCGGTTCGTAGTTCGATGAAAAA
CGGGGCAAAAACCGTTATTTGGCGTGAATTGCAGACATATTGAACGCTAAAATTTGGAA
CGCAAATTGGCACTATCGGGTTTATATCTGATAGTAATGTTGGGTTGAGGGGTCGATTAA
CTCGTGACCTTACAGTCAGCTTGACCGGTTCCTCCTTCGATTAGGTTACTAATGCAAAAG
GGTACCTTTCCGGTAGGACCCCTTAAATTGGCCAAATAGTGGAATGGAAAGGGTAACGC
CTCCACCAATCAAAACGGTAGGGGGCGTTAGGGGCCAGGGCGTGGCTCTTTGGCCAGCC
AACTTGGACGGCTTGGTGCATACATTACTGTTTCCAGAAGTTGGGTTGGTCACAACAAGC
TGTCCTAGTCGAAAGACTAGACGATTCGCACAGTGGATTCGATGTTCTCGAATTCTGCCT
AACTCCATCTCAGCAGGTATCTGATAATATAAAATTTTTT

Appendix 17 Entomopathogenic nematode isolate TK1 best BLASTn hits (close relatives)

Isolate TK1 relatives	Sequence length (Bp)	Max score	E-value	% Identify	Gene bank Accession No.	Country of origin
Steinernema sp.	1012	1027	0.0	92	AY230186	Kenya
Steinernema sp.	1010	822	0.0	87	AY230184	Srilanka
Steinernema sp.	939	488	2e-133	85	KT358812	Tanzania
Steinernema karii	988	488	2e-133	84	AY230173	Kenya
S. ethiopense	739	486	6e-133	83	JN651414	Ethiopia
Steinernema sp.	737	486	6e-133	83	JN651413	Ethiopia
Steinernema sp.	735	486	6e-133	83	JN651412	Ethiopia
S. pwaniensis	939	483	7e-132	85	KT358811	Ethiopia
Steinernema sp.	1060	483	7e-132	85	KC252604	India
Steinernema sp.	911	483	7e-132	85	JF834533	Thailand
S. hermaphroditum	935	477	3e-130	85	MF663703	India
S. lamjungense	815	462	1e-126	87	HM000101	India
S. guangdongense	986	459	1e-124	86	AY170341	China
S. longicaudum	955	353	6e-93	90	AY170337	China
S. jeffreyense	857	318	2e-82	81	KC897093	S. Africa
S. glaseri					AF122015	Belgium
Heterorhbditis safricana	995				EF488006 (Outgroup)	S. Africa

Appendix 18 16S rDNA sequence for bacteria isolate TK1

AGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGCAGCGGGGG
AAAGCTTGCTTTCCTGCCGGCGAGCGGCGGACGGGTGAGTAATGTCTGGGGATC
TGCCCGATGGAGGGGGATAACCACTGGAAACGGTGGCTAATACCGCATAACCTC
TTTGGAGCAAAGTGGGGGACCTTCGGGCCTCACGCCATCGGATGAACCCAGATG
GGATTAGCTAGTAGGTGGGGTAAAGGCTCACCTAGGCGACGATCCCTAGCTGGT
CTGAGAGGATGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGG
AGGCAGCAGTGGGGAATATTGCACAATGAGCGCAAGCCTGATGCAGCCATGCCG
CGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGCA
CAAGGTCGAATACACTGTGCGATTGACGTTACCCACAGAAGAAGCACCGGCTAA
CTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTAC
TGGGCGTAAAGCGCACGCAGGCGGTCAATTAAGTTAGATGTGAAATCCCCGGGC
TTAACCTGGGAATGGCATCTAAGACTGGTTGGCTAGAGTCTCGTAGAGGGGGGT
AGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAATACCGGTGGC
GAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGC
AAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTGGAGG
TTGTGCCCTTGAGCTGTGGCTTCCGGAGCTAACGCGTTAAATCGACCCCCTGGGG
AGTACGGTCGCAAGATTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGG
TGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACAT
CCACGGAATTCGGCAGAGATGCGGAAGTCCCTTCGGGAACCGTGAGACAGGTGC
TGCATGGCGGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAG
CGCAACCCTTATCCTTTGTTGCCAGCACGTTAGGGTGGGAACTCAAGGGAGACTG
CCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTAC
GAGTAGGGCTACACACGTGCTACAATGGCAGATACAAAGAGAAGCGACCTCGCG
AGAGCAAGCGGACCTCATAAAGTCTGTCGTAGTCCGGATTGGAGTCTGCAACTC
GACTCCATGAAGTCGGAATCGCTAGTAATCGCAGATCAGAATGCTACGGTGAAT
ACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAA
AGAAGTCAGGTAGCTTAACCTTTTGGAGGGCGCTGACCATCCTTTGTGATTCATG
ACTGGGGTGAAGTCGTAACAAGGTAACCGTAGGGGAACC

Appendix 19 Bacteria isolate TK1 best BLASTn hits (close relatives)

Bacteria isolate TK1 relatives	Sequence length	Max Score	Query Cover	E value	% Identity	Accession No.	Host EPN
Xenorhabdus sp. MY8NJ	1497	2678	100	0.0	98.93	AB507811.1	*Steinernema* sp. my8
X. ishibashii	1537	2678	100	0.0	98.93	AB243427. 1	*Steinernema aciari*
X. ishibashii Gdh7	1480	2636	98	0.0	98.79	NR117216.1	*Steinernema aciari*
X. eopokensis DL20	1496	2591	99	0.0	98.06	NR156925.1	*Steinernema* sp.
X. sp. GDC328	1480	2575	98	0.0	98.04	Gq149085.1	*Steinernema leizhouense*
X. griffinae IDIO	1473	2562	98	0.0	98.04	NR043643.1	*S. hermaphroditum*
Xenorhabdus sp.VN13	2015	2555	99	0.0	97.46	FJ515800.1	*Steinernema* sp.
X. ehlersii strain Dsm	1524	2545	100	0.0	97.34	NR042327.1	*Steinernema serratum*
X. poinarii NC	2014	2540	100	0.0	97.27	FJ515806.1	*Steinernema glasieri*
Xenorhabdus thuongxuasensis	1526	2534	100	0.0	97.00	NR156924.1	*Steinernema* sp.
Escherichia coli	1541					J01859.1	-

Appendix 20 Effect of bacterial cells in suspension on 2nd instar larvae of *Tuta absoluta* at 24 h exposure (Mean±SEM)

Concentration	Mean±SEM	P-Value
Control 0	0± 0.0d	<0.001
25	68± 4.9c	
50	84± 4.0b	
75	92± 4.9 ab	
100	96 ±4.0 a	

Appendix 21 Variate, Mortality at time 24th h

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Rep	4	480	120	1.71	
Dosage	4	31200	7800	111.43	<.001
Residual	16	1120	70		
Total	24	32800			

Appendix 22 Effect of bacteria cells concentration on 2nd instar larvae of *Tuta absoluta* (48th h)

Concentration	Mean±SEM	P-Value
Control 0	0 ±0.0 b	<0.001
25	88±8.0 a	
50	96±4.0 a	
75	96± 4.0 a	
100	100±0.0 a	

Appendix 23 Comparison between entomopathogenic nematode symbiotic bacteria exposure time and mortality on 2nd instar larvae of *Tuta absoluta*

Exposure time (h)	Mean±SEM	P-value
24	68±7.394 a	0.484
48	76±8.000 a	