# Progress on entomopathogenic nematodes (EPNs) for the control of false codling moth *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae) in avocado orchards in South Africa

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**ABSTRACT**
Entomopathogenic nematodes (EPNs) of the genera *Steinernema* and *Heterorhabditis* occur naturally in soil throughout the world where they parasitise different life stages of various soil-inhabiting insects. The nematodes are symbiotically associated with bacteria and together they kill and utilise their insect host. Infective juveniles (IJs) are the only free-living stage of nematodes found in the soil and carry the bacteria in their intestines, releasing them once the body of the host is penetrated. In addition to being able to kill their hosts within 48 hours, nematodes can be produced commercially and applied with standard spraying equipment or through certain types of irrigation systems. The main interest in these nematodes is their potential as biological control agents in integrated pest management systems.

During a survey conducted in the north-eastern parts of South Africa during 2014/15 to find endemic EPN isolates, several EPN isolates were identified and maintained at the Agricultural Research Council – Institute for Tropical and Subtropical Crops (ARC-ITSC) and the University of Stellenbosch. The aim is to mass rear these isolates to be used in laboratory bioassays and later in field trials. The EPNs are constantly being recycled through mealworm larvae to build up adequate numbers. Bioassays are currently underway and the EPN isolates will be evaluated to determine which of them is the most virulent against the false codling moth. Last instar larvae of the false codling moth are individually exposed to infective juveniles of the isolates in 24 multiwell bioassay plates. Five plates are used per treatment (isolate) and five control plates for each treatment. 13 mm filter disk are placed evenly in 12 wells of each plate. The wells are then individually inoculated with the required concentration of nematodes in 50 µl of water. Control plates receive 50 µl of water only. A final instar false codling moth larvae is then placed in each well. Plates are then covered and placed inside plastic containers lined with moistened paper towels and closed with a lid to maintain high humidity (RH ± 95%). Containers are incubated in the dark at 25°C for 48 hours, after which the mortality of the false codling moth larvae is determined.

**INTRODUCTION**
The false codling moth, *Thaumatotibia leucotreta* Meyrick (Lepidoptera: Tortricidae), is a pest on avocado in all major avocado producing areas (Van den Berg, 2001) (Fig. 1). Relative low rates of infestation are typical of general infestation patterns of these moths on subtropical fruit. However, in a number of cases where conditions are favourable, tortricid

moths are known to become a serious pest (Schoeman & De Beer, 2008). The eggs are oviposited singly on the fruit. Larval entrance holes on the fruit can be spotted by the white exudate and granular excreta (Du Toit *et al*., 1979; Du Toit & De Villiers, 1990). Resulting lesions reduce the market value of fruit due to culling (Fig. 2). Larvae usually do not complete the life cycle in avocado fruit on the tree, but in other subtropical fruit, larvae leave the fruit at pupation (Erichsen & Schoeman, 1992; Newton, 1998; Grove *et al*., 2000). Last instar larvae (Fig. 3) drop to the ground and pupate on the soil surface or beneath leaf litter.

**Figure 1.** The false codling moth, *Thaumatotibia leucotreta* (Meyrick).

**Figure 2.** Feeding damage of false codling moth larvae on avocado fruit. Note the granular excreta of the larvae protruding from the fruit on the right.

**Figure 3.** Final instar larvae of the false codling moth.

The South African avocado industry is interested in gaining access to new markets. The United States Department of Agriculture has conducted a pest risk analysis and identified, among others, false codling moth as a pest of quarantine importance.

If the export market is expanded to new countries, the industry needs to ensure that fruit is false codling moth free, as false codling moth is a quarantine pest for many of the new markets. In South Africa, the avocado industry currently employs a combination of cultural, chemical and microbial control techniques to suppress insect pests like the false codling moth. However, none of these measures target the soil-borne stages of the false codling moth. As soil is the natural habitat of entomophagous nematodes (EPNs), the last instar false codling moth larvae which fall onto the soil, as well as the pre-pupae, pupae and emerging moths, offer a window of opportunity for the use of entomopathogenic nematodes as bio-control agents against this moth pest.

**Entomopathogenic nematodes**

Entomopathogenic nematodes (EPNs) are soil-inhabiting, lethal insect parasitoids. EPNs of the families Steinernematidae and Heterorhabditidae, with their associated symbiotic bacteria, *Xenorhabdus* for Steinernematidae and *Photorhabdus* for Heterorhabditidae, are widely distributed in soils throughout the world (Hominick *et al*., 1996; Hominick, 2002; Adams *et al*., 2006). These nematodes are parasites of insects, killing them within 48 hours, with the aid of their associated bacterial symbiont. Since the late 1970's these nematodes have gained status as one of the best non-chemical alternatives for the control of insect pests, mainly due to their ability to reach insects in cryptic habitats, their reproductive ability, the ease of mass producing them and their safety to humans and other vertebrates (Gaugler, 2007). The infective juvenile EPN is microscopic, 0.5 mm to 1.5 mm long and has a closed mouth and anus and cannot feed until it finds an insect. It enters into the body of the insect through the insect's natural openings – the mouth, anus or respiratory inlets (Poinar, 1990). Once in the blood of the insect, the EPN infective juvenile releases a highly specialised symbiotic bacterium found only in EPNs. These bacteria multiply and rapidly kill the insect. No special methods are required for the application of these nematodes, as they can be applied as an aqueous suspension, using ordinary agrochemical spray equipment.

Various tests against rats, rabbits and monkeys (Gaugler, 1979; Wang *et al*., 1983, 1984; Wang & Lui, 1983; Boemare *et al*., 1996) have shown that the EPNs tested are harmless when fed, injected or inhaled. They are also harmless to earthworms (Capinera *et al*., 1982) and non-target organisms such as plants. They have now been used on a large scale in various countries over the past years and large numbers of production workers have been exposed to thousands of billions of them without any adverse effects being recorded. These biological control agents have proved to be effective against several soil insects and pests that occur in cryptic habitats,

including codling moth on apples and pears (Georgis & Manweiler, 1994; Koppenhöfer, 2000).

**Entomopathogenic nematodes in South Africa**

In South Africa, the first occurrence of a *Steinernema* species, retrieved from the maize beetle in a maize field in Grahamstown, was documented in 1953 (Harrington, 1953). Several years later, in a survey to obtain effective nematodes for the possible control of the African sugarcane stalk-borer, many isolates of both *Heterorhabditis* and *Steinernema* were found (Spaull, 1988, 1990, 1991). De Waal (2008) did a study on the potential of using EPNs on the codling moth, *Cydia pomonella*, under South African conditions and concluded that EPNs can provide effective control of the codling moth. In a survey done by Malan *et al*. (2011) in citrus orchards in the Western Cape, Eastern Cape and Mpumalanga provinces, they identified six species of EPNs as potential biocontrol agents for the false codling moth on citrus. Laboratory bioassays have shown that isolates of all six species found during this survey were highly virulent against the final instar larvae of false codling moth. It also showed that emerging adult moths were infected with nematodes, which may aid in control and dispersal. Few other surveys have been conducted in South Africa (Malan *et al*., 2006; Hatting *et al*., 2009) and throughout the rest of the African continent, which remains relatively unexplored, offering a fertile field for bio-prospecting.

EPNs are commercially available in numerous countries in several formulations (Grewal & Peters, 2005). Concerns with using exotic EPNs include the possible displacement of native nematodes, effects on non-target organisms (Ehlers, 2005) and strict South African regulations regarding the importation of exotic organisms (amendment of Act 18 of 1989 under the Agricultural Pest Act 36 of 1947). Furthermore, exotic nematodes are not adapted to local environmental conditions. Surveys are currently being conducted in many countries, other than South Africa, to find endemic nematode isolates with good efficacy against a specific target insect.

**Objectives of this study**

The main objectives of this study are:

- To isolate EPNs especially from South African macadamia, avocado and litchi orchards.
- To mass rear EPN isolates found in samples for use in laboratory bioassays.
- To determine the potential of the EPN isolates found in these soils for control of the soil stages of the target insect and identifying the most promising isolate by means of bioassays.
- To evaluate most promising EPN isolate in field trials where the effects of concentration, temperature, humidity and other environmental conditions will be determined on the efficacy of the EPNs.

## MATERIALS AND METHODS

**Soil samples**

Soil samples were collected randomly from litchi, macadamia, avocado, other sub-tropical fruit orchards, as well as undisturbed soils in Mpumalanga, Limpopo and KwaZulu-Natal provinces during 2014/15. Each of the soil samples of approximately 2 kg comprised of three sub-samples taken at a depth of up to 20 cm in an area of 3 $m^2$. The samples were placed in polyethylene bags (450 mm x 300 mm) to minimize dehydration. The bags were marked clearly and the GPS points per sample were determined. Other data recorded at each sampling site includes height above sea level, crop, cultivar and age of the trees. The soil samples were transported in an insulated cooler to the laboratory at the Agricultural Research Council - Institute for Tropical and Subtropical Crops (ARC-ITSC) in Nelspruit, Mpumalanga. The samples were initially stored at room temperature in the laboratory and processed within the first week of collection.

**Nematode recovery**

The insect baiting technique (Bedding & Akhurst, 1975) was used to recover the nematodes from the soil. Each soil sample was thoroughly mixed and two 1ℓ plastic containers were each filled with 900 mℓ of soil. Five mealworm (*Tenebrio molitor* (L.)) larvae were placed on the soil surface of each container, covered with a lid and placed in a growth chamber for 7-14 days at 25°C. Thereafter, the dead larvae were removed, rinsed with filtered water and placed on a moistened filter paper in a Petri dish (30 mm x 10 mm). After 2-3 days in the Petri dish, larvae showing signs of infection by EPNs were placed on a modified White trap (White, 1927) for the collection of the emerging infective juveniles (IJs) (Fig. 4). The modified White trap consisted of the bottom part of a Petri dish (85 mm diameter) placed in a glass Petri dish (140 mm diameter) (Fig. 5). The *T. molitor* cadavers were placed on a moist piece of filter paper (80 mm diameter) in the bottom of a plastic Petri dish. The outer glass Petri dish was filled with 20 mℓ filtered water. The IJs crawled into this part soon after emerging from the insect cadavers. Infective juveniles were harvested during the first week of emergence (Fig. 5). The IJs were sent to Dr. Antoinette Malan at the University of Stellenbosch for identification. The rest of each soil sample was sent to the ARC-ITSC soil laboratory for a routine soil analysis.

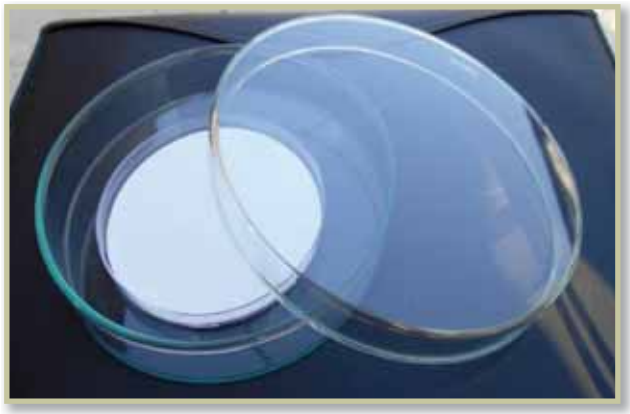

**Figure 4.** A modified White trap consisting of a Petri dish (85 mm diameter) placed in a glass Petri dish (140 mm diameter).

**Figure 5.** Thousands infective juveniles, seen as a milky substance.

**Laboratory bioassays:**

***Twenty-four-well bioassay protocol***

24-well bioassay trays are used as the test arena. To obtain even distribution in the plates, every alternate well is lined with a circular piece of filter paper (13 mm diameter), thus using 12 wells per tray and five trays for each treatment (nematode isolate) and five control plates for each treatment (Fig. 6). Each of the 12 wells is inoculated with a specific concentration of IJs in 50 µℓ filtered tap water. Control plates receive 50 µℓ of water only. A last instar FCM larvae is added to each of the wells (Fig. 7). The wells are then covered with a glass pane inside the lid to prevent the FCM larvae from escaping and secured with a rubber band. The wells are then closed in a plastic container lined with moistened tissue paper (to ensure high relative humidity) and placed in a growth chamber at 25 ± 2°C for 48 hours. Thereafter, mortality is determined and infection of the insects is confirmed by dissection of insects with the aid of a stereomicroscope.

**Figure 6.** 12 wells in a 24-well bioassays plate lined with filter paper.

**Figure 7.** Last instar false codling moth larvae is placed in each of the 12 wells.

***Source of insects***

Mealworm, *Tenebrio molitor* (L) (Coleoptera: Pyralidae), last instar larvae is reared at room temperature in plastic containers on fine wheat bran. To improve humidity, apple slices are laid over the surface of the colony. Last instar mealworm larvae is harvested regularly and kept at 4°C until needed.

***Source of nematodes***

Nematode isolates in this study are obtained from the survey that was done during 2014/15 and maintained at the University of Stellenbosch and the ARC-ITSC. For each of the bioassays, nematode inoculum is freshly prepared, using *Tenebrio molitor* (meal worm) larvae. Nematodes are harvested within the first week of emergence from the modified white traps (White, 1927), and stored horizontally at 14°C, in 500 mℓ vented culture flasks and used within one month after harvesting. The flasks are shaken weekly to improve aeration and nematode survival. Nematode concentrations are calculated according to the technique of Navon and Ascher (2000).

## RESULTS AND DISCUSSION

**Soil samples**

During the survey the focus was to take soil samples on subtropical crops where Lepidopteran pests are a problem. Samples were therefore taken from avocado, litchi and macadamia orchards, as well as undisturbed soils in the production areas of these crops in the north eastern parts of the country. A total of 136 soil samples were taken from the different production areas: 38 from Mpumalanga, 57 from Limpopo and 41 from KwaZulu-Natal. A few samples still need to be taken from the KwaZulu-Natal avocado producing areas. Of the total of 136 samples, 13 of the samples tested positive for EPNs. This represents a 9.6% recovery rate. Positive samples are currently at the University of Stellenbosch for DNA identification of the species. EPN isolates are also kept at the ARC-ITSC in Nelspruit for laboratory bioassays.

**Laboratory bioassays**

Since this part of the study only started in January 2016, no data is yet available on the bioassays.

This EPN survey was the first systematic survey conducted to assess the presence and diversity of EPNs occurring on a specific crop or crop type in the north-eastern parts of South Africa (Mpumalanga, Limpopo, and KwaZulu-Natal). The few other surveys done in South Africa, focused mainly in the Western Cape province (De Waal, 2008; Hatting *et al*., 2009; Malan *et al.*, 2006; Malan *et al*., 2011) and on different crops such as apples, pears and citrus.

Soil samples during the survey were also taken from undisturbed natural soils. In contrast to human modified areas, natural habitats are more likely uncontaminated by introduced nematodes and therefore offer a better chance for finding native species.

Research into the biological control of insects has shown that no single biocontrol method, including the use of EPNs can, by itself, effectively replace pesticide usage. Research into EPNs in South Africa has mostly been directed toward the control of insect pests on a commercial scale. To integrate nematodes into an integrated pest management system, it is important to conduct research under local climatic conditions for a specific crop. Especially for commercial application, the unique environmental conditions in the various production areas need to be assessed to allow for the effective use of various nematode species. Research into endemic EPNs, mainly targeting the two key South African lepidopteran pests, codling moth (*Cydia pomonella*) on apples and pears, and false codling moth (*Thaumatotibia leucotreta*) on citrus, forms the current bulk of our knowledge.

## FUTURE RESEARCH

- Conclude soil sampling in the production areas of KwaZulu-Natal province.
- Continue with bio-assays in laboratory to determine the potential of EPN isolates found and identifying the most virulent isolate.
- Evaluate the most virulent isolate in field trials together with the commercially available product from River Bioscience to determine the efficacy of these EPNs on false codling moth in avocado orchards.


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## REFERENCES


ADAMS, B.J., FODOR, A., KOPPENHôFER, H.S., STACKEBRANDT, E., STOCK, S.P. & KLEIN, M.G. 2006. Biodiversity and systematics of nematode-bacterium entomopathogens. *Biological Control* 37: 32-49.

BEDDING, R.A. & AKHURST, R.J. 1975. A simple technique for detection of insect parasitic rhabditid nematodes in soil. *Nematologica* 21: 109-110.

BOEMARE, N., LAUMOND, C. & MAULEON, H. 1996. The Entomopathogenic nematode-bacterium complex: biology, life cycle and vertebrate safety. *Biocontrol Science and Technology* 6(3): 333-345.

CAPINERA, J.L., BLUE, S.L. & WHEELER, G.S. 1982. Survival of earthworms exposed to *Neoaplectana carpocapsae* nematodes. *Journal of Invertebrate Pathology* 39(3): 419-421.

DE WAAL, J.Y. 2008. Entomopathogenic nematodes (Rhabditidae: Steinernematidae and Heterorhabditidae) for the control of Codling moth, *Cydia pomonella* (L.) under South African conditions. M.Sc. Dissertation, Stellenbosch University, Stellenbosch.

DU TOIT, W.J., DE VILLIERS, E.A. & TUFFIN, A. 1979. The identification of causes of typical surface lesions on avocado fruit. *South African Avocado Growers' Association Yearbook* 3: 52-53.

DU TOIT, W.J. & DE VILLIERS, E.A. 1990. Identifisering van avokadovrugletsels wat deur insekte veroorsaak word. *South African Avocado Growers' Association Yearbook* 13: 56-60.

EHLERS, R.U. 2005. Forum on safety and regulation. *In* Grewal, P.S., Ehlers, R. U., Shapiro-Ilan, D.I. (eds.), Nematodes as biocontrol agents. CABI Publishing, Wallingford, UK, 107-114.

ERICHSEN, C. & SCHOEMAN, A. 1992. Economic losses due to the insect pests on avocado fruit in the Nelspruit/Hazyview region of South Africa during 1991. *South African Avocado Growers' Association Yearbook* 15: 49-54.

GAUGLER, R. 1979. The entomogenous nematode *Neoaplectana carpocapsae*: radiation and mammalian safety studies. *Dissertation Abstracts International* 40B (3): 1050-1051.

GAUGLER, R. 2007. Entomopathogenic Nematology. CABI Publishing, Wallingford, UK.

GEORGIS, R. & MANWEILER, S.A. 1994. Entomopathogenic nematodes: a developing biological control technology. *Agricultural Zoological Reviews* 6: 63-94.

GREWAL, P.S. & PETERS, A. 2005. Formulation and quality. *In* Grewal, P.S., Ehlers, R.U., Shapiro-Ilan, D.I. (eds.), Nematodes as biocontrol agents. CABI Publishing, Wallingford, UK, 79-90.

GROVé, T., STEYN, W.P. & DE BEER, M.S. 2000. Host status of Hass avocado fruit for the false codling moth, *Cryptophlebia leucotreta* (Meyrick) (Lepidoptera: Tortricidae). *South African Avocado Growers' Association Yearbook* 23: 99-102.

HARRINGTON, J.S. 1953. Observation on the biology, the parasites and the taxonomic position of the maize-beetle – *Heteronychus sanctae-helenae* Blanch. *South African Journal of Science* 50: 11-14.

HATTING, J., STOCK, P.S. & HAZIR, S. 2009. Diversity and distribution of Entomopathogenic nematodes (Steinernematidae, Heterorhabditidae) in South Africa. *Journal of Invertebrate Pathology* 102: 120-128.

HOMINICK, W.M., REID, A.P., BOHAN, D.A. & BRISCOE, B.R. 1996. Entomopathogenic nematodes: biodiversity, geographical distribution and the Convention on Biological Diversity. *Biocontrol Science and Technology* 6: 331.

HOMINICK, W.M. 2002. Biogeography. *In* Gaugler, R. (ed.), Entomopathogenic Nematology, CABI Publishing, Wallingford, UK, 115-143.

KOPPENHöFER, A.M. 2000. Nematodes. In: Lacey, L.A., Kaya, H.K. (eds.), Field manual of techniques in invertebrate pathology. Kluwer Academic Publishers, The Netherlands, 283-301.

MALAN, A.P., NGUYEN, K.B. & ADDISON, M.F. 2006. Entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) from the southwest parts of South Africa. *African Plant Protection* 12: 65-69.

MALAN, A.P., KNOETZE, R. & MOORE, S.D. 2011.

Isolation and identification of entomopathogenic nematodes from citrus orchards in South Africa and their biocontrol potential against the false codling moth. *Journal of Invertebrate Pathology* 108: 115-125.

NAVON, A. & ASCHER, K.R.S., 2000. Bioassays of Entomopathogenic microbes and nematodes. CAB International, Wallingford, UK, 229-247.

NEWTON, P.J. 1998. False codling moth *Cryptophlebia leucotreta* (Meyrick). pp.192-200. *In* Bedford, E.C.G., Van Den Berg, M.A. & De Villiers, E.A. (Eds) *Citrus Pests in the Republic of South Africa.* Dynamic Ad, Nelspruit. p.288.

POINAR, G.O. 1990. Taxonomy and biology of Steinernematidae and Heterorhabditidae. *In* Gaugler, R., Kaya, H.K. (eds.), Entomopathogenic nematodes in biological control. CRC Press, Boca Raton, 23-61.

SCHOEMAN, P.S. & DE BEER, M.S. 2008.Control of the false codling moth with Isomate mating disruption technology. *South African Avocado Growers' Association Yearbook* 31:42-43.

SPAULL, V.W. 1988. A preliminary evaluation of entomogenous nematodes to control the African sugarcane stalk borer *Eldana saccharina* (Lepidoptera: Pyralidae). *Proceedings of the South African Sugar Technologists Association* 62: 120-123.

SPAULL, V.W. 1990. Field test to control the pyralid, *Eldana saccharina*, with an entomogenous nematode, *Heterorhabditis* sp. *Proceedings of the South African Sugar Technologists Association* 64: 103-106.

SPAULL, V.W. 1991. *Heterorhabditis* and *Steinernema* species (Nemata: Rhabditida) for the control of a sugar cane borer in South Africa. *Phytophylactica* 23: 213-215.

VAN DEN BERG, M.A. 2001. False codling moth, *Cryptophlebia leucotreta* (Meyrick). In: Van den Berg, M.A., De Villiers, E.A. & Joubert. P.H. (Eds.) *Pests and Beneficial Arthropods of Tropical and Non-citrus Subtropical Crops in South Africa*. ARC-Institute for Tropical and Subtropical Crops, Nelspruit, p. 320-325.

WANG, J.X. & LUI, Z.M. 1983. The safety of the nematode, *Neoaplectana glaseri* Steiner, to invertebrates. II. A test on rabbits. *Natural Enemies of Insects* 5(4): 240-242.

WANG, J.X., QUI, L.H. & LUI, Z.M. 1983. The safety of the nematode *Neoaplectana glaseri* Steiner to vertebrates. I. A test on rats. *Natural Enemies of Insects* 5(1): 39-41.

WANG, J.X., HUANG, J.T. & CHEN, Q.S. 1984. The safety of the nematode *Neoaplectana glaseri* Steiner to vertebrates. III. A test on monkeys, *Macaca mulatta*. *Natural Enemies of Insects* 6(1): 41-42.

WHITE, G.F. 1927. A method for obtaining infective nematode larvae from culture. *Parasitology* 4: 147-154.