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Isolation and identification of entomopathogenic nematodes from citrus orchards in South Africa and their biocontrol potential against false codling moth

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ABSTRACT

A survey was conducted to determine the diversity and frequency of endemic entomopathogenic nematodes (EPN) in citrus orchards in the Western Cape, Eastern Cape and Mpumalanga provinces of South Africa. The main aim of the survey was to obtain nematodes as biological control agents against false codling moth (FCM), *Thaumatotibia leucotreta*, a key pest of citrus in South Africa. From a total of 202 samples, 35 (17%) tested positive for the presence of EPN. Of these, four isolates (11%) were found to be steinernematids, while 31 (89%) were heterorhabditids. Sequencing and characterisation of the internal transcribed spacer (ITS) region was used to identify all nematode isolates to species level. Morphometrics, morphology and biology of the infective juvenile (IJ) and the first-generation male were used to support molecular identification and characterisation. The *Steinernema* spp. identified were *Steinernema khoisanae*, *Steinernema yirgalemense* and *Steinernema citrae*. This is the first report of *S. yirgalemense* in South Africa, while for *S. citrae* it is the second new steinernematid to be identified from South Africa. *Heterorhabditis* species identified include *Heterorhabditis bacteriophora*, *Heterorhabditis zealandica* and an unknown species of *Heterorhabditis*. Laboratory bioassays, using 24-well bioassay disks, have shown isolates of all six species found during the survey, to be highly virulent against the last instar of FCM larvae. *S. yirgalemense*, at a concentration of 50 IJs/FCM larva caused 100% mortality and 74% at a concentration of 200 IJs/pupa. Using a sand bioassay, *S. yirgalemense* gave 93% control of cocooned pupae and emerging moths at a concentration of 20 IJs/cm^2. This is the first report on the potential use of EPN to control the soil-borne life stages of FCM, which includes larvae, pupae and emerging moths. It was shown that emerging moths were infected with nematodes, which may aid in control and dispersal.

1. Introduction

The false codling moth (FCM), *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae), occur naturally in South Africa and also in sub-Saharan Africa and the Indian Ocean islands (CABI/EPPO, 1976). It is a serious pest of citrus in South Africa, and can cause major economic losses (Moore et al., 2004). It is also a pest of phytosanitary concern, with restrictive import regulations having been imposed by importing countries (Bloem et al., 2003) such as the USA. During 2008, a total of 58,102 ha of citrus were cultivated in all provinces of South Africa, except for the Free State. The majority of citrus planted is in Limpopo, the Eastern Cape, Mpumalanga and the Western Cape provinces (CGA, 2010). In South Africa, citrus is a major export-based industry and represents a huge investment in both foreign exchange earnings and human resources.

The life cycle of FCM is 25–60 days and up to eight generations per year have been recorded in South Africa (Stofberg, 1954; Georgala, 1969; Daiber, 1980). The moths lay their eggs on the fruit. The hatched larvae penetrate the fruit, causing premature fruit drop (Daiber, 1989). The last-instar FCM larvae drop with a silken thread onto the soil, in which they bury themselves under a few millimetres, and spin tightly woven cocoons in which soil is incorporated (Daiber, 1979a). After a period of approximately 2–3 days the prepupa in the cocoon changes into a pupa, and the adult moth emerges after a further 12–16 days at 25 °C, with longer intervals at lower temperatures (Daiber, 1979b).

Entomopathogenic nematodes (EPN) of the families Steinernematidae and Heterorhabditidae, with their associated symbiotic bacteria, 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 h with the aid of their associated bacterial symbiont. Since the late 1970s, these nematodes have gained status as one of the best non-chemical alternatives for the control of insect pests, mainly

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due to their ability to reach insects in cryptic habitats, their high reproductive ability, the ease of mass producing them, and their safety to humans and other vertebrates (Gaugler, 2007).

In South Africa, the citrus industry currently employs a combination of cultural, chemical and microbial control techniques to suppress FCM. The methods employed include orchard sanitation (Moore and Kirkman, 2008), mating disruption (Hofmeyer et al., 2005), and biological control, using a *Cryptophlebia leucotreta* granulovirus, as well as the sterile insect technique (Hofmeyer et al., 2005). However, none of the control measures named target the soil-borne stages of FCM. As soil is the natural habitat of EPN, the last-instar FCM larvae which fall onto the soil, as well as the prepupae, pupae and emerging moths, offer a window of opportunity for the use of nematodes as biological control agents against FCM. Nematodes can also fill an important niche in early spring, summer, autumn, and after harvest, when, traditionally, no control measures are implemented.

Concerns with using exotic EPN 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 (Grewal et al., 2001). 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, thereby circumventing the importation of exotic nematodes (Grewal et al., 2001).

Few 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 bioprospecting. Recent surveys from Africa revealed new steinernematids such as *Steinernema karii* from Kenya (Waturu, 1998), *Steinernema taysaerae* from Egypt (Shamseldean et al., 1996), and *Steinernema yirgalemense* from Ethiopia (Nguyen et al., 2004). In Kenya, new strains of *Heterorhabditis bacteriophora* and *Heterorhabditis indica* have been isolated. Isolates of *S. yirgalemense, S. karii* and *Steinernema weiseri* have also been reported from the Central Rift Valley Region of Kenya (Mwaniki et al., 2008). In Ethiopia, the dominant species detected was *S. yirgalemense* (6.3%), with only two isolates of *H. bacteriophora* (0.7%) (Mekete et al., 2005). Three new species, *Steinernema khoisanae* (Nguyen et al., 2006), *S. citrae* (Stokwe et al., 2011) and *Heterorhabditis safricana* (Malan et al., 2008), have recently been described for South Africa.

The primary objective of the current study was to obtain EPN specifically from South African citrus orchards and to contribute to the existing knowledge of the geographical distribution and diversity of EPN. No previous research has been undertaken into the control of FCM using nematodes and therefore the second objective of the study was to determine the potential of EPN for the control of the soil stages of FCM. Nematode species found during the survey were tested for infectivity by evaluating the percentage mortality in laboratory bioassays against last-instar larvae, pupae and emerging moths.

2. Materials and methods

2.1. Soil sampling, trapping and maintenance of nematodes

Soil samples were collected from citrus orchards in three provinces of South Africa, namely the Western Cape, the Eastern Cape and Mpumalanga. Commercial farms were large, averaging several dozen hectares in size, while individual orchards were usually in the region of a hectare or more. Subsamples were taken from a depth of up to 20 cm, by using a hand spade, from underneath the canopy of four trees, situated in each quadrant of an orchard, and the 16 subsamples from the orchard were combined to form one composite sample of approximately 1 kg.

Nematodes were recovered from the soil samples by using the insect-baiting technique (Bedding and Akhurst, 1975). All the soil from each sample was split up into 250-ml plastic containers. Five larvae of either the greater wax moth, *Galleria mellonella* (L.) (Lepidoptera: Phyralidae) and/or the mealworm, *Tenebrio molitor* (L.) (Coleoptera: Tenebrionidae) were placed on the soil surface of each container, which were then closed. The two trapping-hosts were added together or the one followed by the other, depending on the availability of the hosts. During a 7-day period for wax moth and a 14-day period for mealworm, the samples were periodically checked for the presence of dead insects. Possible EPN-infected cadavers were placed on modified White traps (Kaya and Stock, 1997). Nematodes were harvested within the first week of emergence and used to inoculate wax moth larvae, in order to confirm Koch's postulates for pathogenicity (Steyn and Cloete, 1989). Nematode isolates were maintained at Stellenbosch University in 150 ml of filtered tap water in vented culture flasks, which were kept horizontally at 14 °C and shaken weekly. Infective juveniles (IJs) were maintained by recycling through wax moth larvae every 3 months for heterorhabditids, and every 6 months for steinernematids (Dutky et al., 1964; Nguyen, 1988).

2.2. Molecular characterisation of nematodes

For DNA extraction, the technique described by Nguyen (2007) was used. One first generation female for steinernematids or one hermaphrodite for heterorhabditids, was placed in 30 ml lyses buffer (16 mM $[NH_4]_2SO_4$, 67 mM Tris–HCl pH 8.8, 0.1% Tween-20) containing 60 μg/ml Proteinase K on the side of a 0.5 μl Eppendorf tube. The nematode was cut into pieces with the sharp side of a syringe needle and immediately put on ice and frozen overnight at −80 °C. The Eppendorf tubes were put in a thermocycler at 65 °C for 1 h followed by 95 °C for 10 min and then centrifuged for 2 min at 12 000 rpm. The top 20 μl was transferred to a clean 0.5 μl Eppendorf tube and kept at −20 °C.

The 18S and 26S primers (Whitehead Scientific), suggested by Vrain et al. (1992), were used for amplification of the ITS region. If a good sequence could not be obtained, the primers TW81 and AB28 (Hominick et al., 1997) were used. The technique of Nguyen (2007) for PCR amplification was followed. Purified DNA was sequenced at the Analytical Centre of the Department of Genetics at Stellenbosch University, using the BigDye 3.1 chemistry (PE Applied Biosystems). The base-pair calls of the sequences were verified and edited, using the software CLC DNA Workbench, version 6.

To indicate the phylogenetic position of the nematode isolates found during the survey, sequences of *Heterorhabditis* and *Steinernema* isolates were compared to sequences from Genbank. Phylogenetic and molecular analyses were conducted based on maximum parsimony (MP) of the ITS region using the software ClustalX ver. 1.83 (Thompson et al., 1997) and PAUP* ver. 4.08b (Swofford, 2002) or maximum likelihood (ML) using Mega5 (Tamura et al., submitted for publication).

2.3. Morphometrical characterisation

For morphometrics, 25 IJs and 20 first generation males for *Steinernema* and second generation males for *Heterorhabditis* of an isolate of each species were randomly selected from different infected larvae. Nematodes were killed with hot (85 °C) triethanolamine-formalin (TAF) (Courtney et al., 1955) fixative, and processed to anhydrous glycerine for mounting (Seinhorst, 1959). Measurements were taken, using a Leica DM2000 research