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Abstract

Bactrocera dorsalis fruit fly is the economically most significant tephritid pest species on Mango, *Mangifera indica L*., in Benin, and entomopathogenic nematodes (EPNs) represent good candidates for its control in the soil. In this study, the susceptibility of larvae and pupae of *B. dorsalis* to 12 EPN isolates originating from Benin was investigated. The effect of nematode concentrations (20, 50, 100, 200 and 300 Infective Juveniles (IJs)/ *B. dorsalis* larva) and of different substrate moisture content (10, 15, 20, 25 and 30% v/w) on *B. dorsalis* mortality at the larval stage was studied. Also, the reproduction potential inside *B. dorsalis* larvae was assessed. Our results revealed that the susceptibility of *B. dorsalis* larvae was significantly different among the 12 tested nematode isolates with *H. taysearae* isolate Azohoue2 causing the greatest insect mortality (96.09±1.44%). The lowest insect mortality (7.03±4.43%) was recorded with *Steinernema* sp. strain Bembereke. Significant differences in insect mortality were recorded when EPNs were applied at varying IJs concentrations. A concentration of 100 nematodes of either *H. taysearae* Azohoue2 or *H. taysearae* Hessa1 per *B. dorsalis* larva was enough to kill at least 90% of *B. dorsalis* larvae. Larvae were less susceptible to nematodes at higher moisture content (25% and 30%). In addition, pupae were less susceptible to nematodes than larvae. Furthermore, the tested nematode isolates were able to reproduce inside *B. dorsalis* third instar larva or pupa with the *Heterorhabditis* isolates giving the greatest multiplication rate (59577.2 IJs ± 14307.41).

Keywords: Biological control; tephritid; *Heterorhabditis taysearae*; *Steinernema* sp.; *Mangifera indica* L.

1. Introduction

Mango (*Mangifera indica* L., Anacardiaceae) is one of the most important tropical fruits produced in West Africa, a region most favorable for fruit production and export (Vannière et al., 2004; Gerbaud, 2007; Vayssières et al., 2009a). Mango fruit constitutes a very important source of nutrition for rural populations living in northern Benin (Vayssières et al., 2008). In Africa and particularly in Benin, the production of this fruit is confronted with several problems including quality loss due to fruit flies (Tephritidae, Diptera), especially *Ceratitis capitata, Ceratitis cosyra* and *B. dorsalis* (Vayssières et al., 2009b). The latter, formerly known as *Bactrocera invadens* (Schutze et al., 2014), is the most important pest causing serious damage in orchards of mango as well as in other important tropical fruit crops including guava and citrus (Goergen et al., 2011; Vayssières et al., 2009b). Chemical applications have been used as traditional methods to control these fruit flies for many years. For example, Spinosad GF-120 (Spinosad + foodstuff attractant) and Proteus 170 O-TEQ (Thiaclopride + Deltamethrine) showed great performance for control of flies (Vayssière et al., 2009a; N'Depo et al., 2015). However, the environmental side- effects have led to interest in other, environmental friendly, cost effective and locally available control strategies to inhance mango production and export. In this respect, several control methods have recently been developped including the sterile insect technique (Clarke et al., 2011) and the biological control based on the use of weaver ants, *Oecophylla smaragdina* and *Oecophylla longinoda*, (Anato et al., 2015; Offenberg et al., 2013; Wargui et al., 2015). Unfortunatly, the latter method is associated with some constraints as the ants delay the labor during harvest and are responsible for small black spots left on the fruit (Sinzogan et al., 2008).

EPNs of the genera *Steinernema* (Panagrolaimomorpha: Steinernematidae) and *Heterorhabditis* (Rhabditomorpha: Heterorhabditidae) are effective biocontrol agents (Grewal et al., 2005). They have been found in most countries and are successfully used to control many insect pests around the world (Ehlers, 2001). Several strains *of Heterorhabditis taysearae*,

Heterorhabditis indica and *Steinernema* sp. have been isolated from Benin and all demonstrated a cruiser type insect search strategy (Zadji et al. 2014b). *H. taysearae* Shamseldean, Abou El-Sooud, Abd-Elgawad and Saleh, 1996, has been recently considered as a senior synonym of *Heterorhabditis sonorensis* Stock, Rivera-Orduño and Flores-Lara, 2009 by Hunt and Subbotin, (2016).

The Infective Juvenile (IJ) represents the only free-living developmental stage of EPNs that occurs naturally in the soil. They are symbiotically associated with bacteria of the family *Enterobacteriaceae* which belong to the genera *Xenorhabdus* (*Steinernema*) or *Photorhabdus* (*Heterorhabditis*) (Ciche et al., 2006). IJs of both genera *Steinernema* and *Heterorhabditis* can infect the insect larvae via body openings such as anus, mouth or spiracles (Campbell and Lewis, 2002). In addition to these ways of penetrating the insect host, *Heterorhabditis* species are able to actively enter the hemocoel through the host cuticle by the use of their additional dorsal tooth to perforate the inter-segmental membrane of the cuticle (Bedding and Molyneux, 1982; Griffin et al. 2005). Inside the host they release intestinal bacteria into the insect hemocoel. These bacteria reproduce and produce metabolites that kill the insect within 1-2 days (Dowds and Peters, 2002) and serve at the same time as food source for the nematode. An effective sustainable *B. dorsalis* management approach could be the use of EPNs to control insect pests at soil-borne stages of the insect life cycle. Indeed, the late larval instar of *B. dorsalis* leaves the infested fruit and falls on the ground where it burrows in the top 4 cm of the soil prior to pupating after a short dispersal period (Hou et al., 2006). Adult flies emerge from pupae after 1-2 weeks (longer in cool conditions). This offers an opportunity to EPN IJs present in the soil to invade *B. dorsalis* larvae or pupae even if the exposure time to the larvae is relatively short. Many studies have been conducted on the Mediterranean fruit fly *Ceratitis capitata* (Gazit et al., 2000; Lindegren and Vail, 1986; Lindegren et al., 1990; Malan and Manrakhan, 2009; Minas et al., 2016; Poinar and Hislop, 1981), the Queensland fruit fly *Bactrocera tryoni* (Froggatt) (Langford et *al.,* 2014), the cherry fruit fly *Rhagoletis cerasi L*. (Herz

et al., 2006), *Bactrocera oleae* (Sirjani et al., 2009), *Bactrocera cucurbitae*, *B. dorsalis* (Lindegren and Vail, 1986) and the Natal fruit fly *Ceratitis rosa* (Malan and Manrakhan, 2009) and have demonstrated that the flies were highly susceptible to *Steinernema* and *Heterorhabditis* nematodes.

Based on these previous studies and their known biocontrol abilities, EPN of the families Heterorhabditidae and Steinernematidae in association with their symbiotic bacteria *Photorhabdus* and *Xenorhabdus* respectively, are considered to be promising biocontrol candidates against *B. dorsalis* on mango trees in Benin.

Several studies have revealed that indigenous EPNs are well adapted to local environmental conditions and therefore considered as good biological agents to control insect pests (Bedding, 1990; Grewal et al., 1990; Noujeim et al., 2015; Zadji et al., 2014b). To our knowledge, the susceptibility of *B. dorsalis* to Beninese EPNs has not yet been investigated. The current study is one of a series anticipated for the implementation of cost-effective *B. dorsalis* management using EPNs in mango orchards in Benin. It aimed to: (i) investigate the occurrence of EPNs in mango orchards in northern Benin, (ii) identify the recovered EPN isolates, (iii) test their pathogenicity against mango fruit fly (*B. dorsalis*) under laboratory conditions. Specifically, 12 EPN isolates from Benin were screened for their virulence against the third instar larvae of *B. dorsalis* and the most virulent isolates were selected to investigate the susceptibility of larvae and pupae of *B. dorsalis* under different abiotic laboratory conditions.

2. Materials and methods

2.1. Source of insects

B. dorsalis used in this study were obtained from laboratory rearing initiated from *B. dorsalis* pupae provided by IITA-Benin (International Institute of Tropical Agriculture-Benin). The original colony of *B. dorsalis* used at the IITA- Benin institute was established from naturally infested

mango fruits collected in Northern Benin. Flies were fed with a mixture of brown sugar and yeast extract at 3:1 proportion (Vayssières et al., 2015). Cages were supplied with water. Ripened papaya fruits were exposed to 10 day old female flies to allow them laying eggs into the ripened papaya used as host. The infested papaya was incubated at 28°C and 60–80% relative humidity (RH) during 7 days, after which the third instar of *B. dorsalis* larvae started to exit the fruit. We used in our assays the third instar larvae collected approximately 1 hour after they had jumped from infested papaya to pupate. Larvae that were not used in assays were left in sand with 10% humidity to pupate and become adults within approximately eight days.

2.2. Source of nematodes

Most of the nematodes used in this study were provided by the Laboratoire de Phytotechnie, d'Amélioration et de Protection des Plantes (LaPAPP), Benin. They were collected from soil in several vegetations (Table 1) in Benin (Zadji et al., 2013). Other nematodes were newly collected from a local soil sampling (January- February 2015) exclusively in several mango orchards located in northern Benin. Seventy soil samples in total were collected from fourteen mango orchards (each at least 1 hectare of area) selected at random in eight villages of Parakou, Borgou department located in northern Benin. In each orchard, 5 samples of approximately 1.5 kg each were taken randomly at ≤ 15 cm depth. Each soil sample was individually processed for nematode extraction using the *Galleria mellonella* (Lepidoptera, Piralidae) baiting method (Bedding and Akhurst, 1975) and white trap (White, 1927). Pathogenicity of the isolated nematodes was confirmed by re-infesting fresh *G. mellonella* larvae as described above and newly emerged IJs collected from white trap were kept at 13°C for further study.

The nematode species, sample number, origin, vegetation and accession numbers of all EPN isolates included in this study are presented in Table 1. Nematodes used for the assays were

acclimated for 2 hours at room temperature (25°C) after removal from incubator (13°C) to help them adjust to the new temperature and allow better performance. Nematode viability (based on their movement) was checked under a stereomicroscope. The concentrations of nematodes were calculated by volumetric dilutions in tap water using the formula of Navon and Ascher (2000).

2.3. Nematode identification

The identity of most of the nematode isolates provided by the LaPPAP laboratory was described by Zadji et al. (2013). However, new nematode isolates retrieved from soil samples collected in mango orchards were identified in this study (Table1).

2.3.1. Molecular identification

For each nematode isolate, DNA was extracted from a single specimen in an Eppendorf tube (250 µl) containing 1 µl of double distilled water. Ten µl of 0.05 N NaOH was added plus 1 µl of 4.5% Tween 20 solution (Janssen et al., 2016). The tube was heated at 95°C for 15 min and cooled at room temperature prior to storage at 4°C for use within next month or at -20°C for later use. The ITS region was amplified and sequenced using the primers pair AB28 (ATATGCTTAAGTTCAGCGGGT) and TW81 (GTTTCCGTAGGTGAACCTGC). ITS sequences were aligned with their closest BLAST search matches (obtained from GenBanck database) using ClustalW Multiple alignment. Afterwards, a phylogenetic tree was generated in Mega-6 software using the Neighbor-Joining method (Saitou and Nei, 1987). *Caenorhabditis elegans* EU131007 was used as outgroup.

2.3.2. Morphological/morphometric identification.

Light microscopic pictures were taken using a Soft Imaging System GmbH (Cell^D software, Münster-Germany) connected to an Olympus BX51 microscope. The same system was used to

measure 20 IJs and, 20 F1 males of the studied nematode strains. Juveniles were heat killed and mounted on temporal slides while males were fixed and mounted on permanent slide for measurements.

2.3.3. Cross breeding

To confirm the reproduction compatibility of the new *Heterorhabditis* isolates with described ones, cross hybridization tests were performed on lipid agar (Wouts, 1981) according to the method of Phan et al. (2003). Indeed, it was assumed that mating between male and female of the same species should produce fertile offspring (Nguyen, 2007). Crossings were restricted to the newly isolated *Heterorhabditis* nematode strains and *H. taysearae*Hessa1, described by Zadji et al. (2013). Twenty males and 20 virgin females of the appropriate nematode strains were crossed. Controls consisted of incubating 20 virgin females without males (virginity test) and 20 males x 20 females of the same isolate (self-cross test). Plates were incubated at 25°C for 2-4 days after which the presence of juveniles was examined. Results were considered valid only when the self-cross test was positive and the virginity test negative.

2.4. Pathogenicity tests

2.4.1. Screening of insect mortality induced by nematode isolates

Twenty four well plates were used. Each well (3.14 cm^2 surface area) was filled with 1 ml of heat-treated (80°C, 72 h) sand (grain size < 2 mm). One hundred IJs suspended in 200µl of tap water were transferred into each well in order to obtain 20% (v/w) moisture content. Controls received only 200 µl tap water (without nematodes). Thereafter, one third instar *B. dorsalis* larva was placed on top of the sand in each well to allow them to burrow in the sand naturally. Plates were arranged in a completely randomized block design with three replications (a plate with 24 wells represented one replicate for each EPN isolate or the control), and kept in dark at 28°C. A

replicate (bloc) was consisted of 13 treatments (12 isolates plus one control). After 48 hours of incubation, insects were retrieved from the sand of individual wells. The number of dead larvae was recorded and pupae from the same twenty four well plate were transferred into a small plastic container (7 cm diameter x 5.5 cm height). The plastic container was covered with a perforated lid to allow aeration before being kept at 28°C. After 14 days, emerged flies as well as unemerged pupae were recorded. We hypothesized that after 14 days the pupae that had not developed into adult (flies) had been killed by nematodes. Therefore, at most five dead insects (larvae and unemerged pupae) were randomly selected and individually dissected after being kept at room temperature for 48 hours to ascertain their infection by nematodes.

The number of dead larvae was added to that of unemerged pupae to determine insect mortality. The experiment was repeated twice with different batches of nematodes. All nematode isolates in Table 1 were involved in this study.

2.4.2. Effect of nematode concentrations on *Bactrocera dorsalis* mortality

H. taysearae isolates Azohoue2 and Hessa1 and *Steinernema* sp. isolate Thui were selected to examine the effect of their concentration on *B. dorsalis* mortality because they induced higher insect mortality among isolates of their species in the screening experiment (2.4.1). The isolate *H. indica* Ayogbe1 which also induced a higher insect mortality was not included because it was contaminated by fungi during the experiment and has been discarded. The experiment arena consisted of a 24-well plate as described above. Different nematode concentrations of 20, 50, 100, 200 and 300 IJs/ well corresponding to 6, 16, 32, 64 and 95 IJs/cm2 respectively, were tested at 20% (v/w) moisture content. Controls received only 200 µl tap water (without nematodes). Three plates (replicates) were used per treatment (isolate x concentration). They were arranged in a completely randomized block design with all plates of the same replicate representing each bloc. The experiment was repeated twice with different batches of the three

nematode isolates. Insect mortality (larvae and pupae) was determined in the same conditions as described above. At most five dead insects were randomly selected and individually dissected to confirm death by nematodes.

2.4.3. Effect of soil moisture on the pathogenicity of EPN to *Bactrocera dorsalis*

Two isolates of *H. taysearae* (Azohoue2 and Hessa1) and one of *Steinernema* sp. (Thui) were used to examine the effect of soil moisture on insect mortality. Different soil moistures were tested to determine the optimal soil moisture content that is conducive to the nematode isolates to control *B. dorsalis.* Sandy loam soil was heat-treated as above mentioned and wetted to reach the final moisture content (v/w) of 10, 15, 20, 25 and 30% including the water added with the nematode suspension. Nematodes were applied at 100 IJs per *B. dorsalis* larva as described above and plates were incubated in dark at 28°C for 48 hours. The experiment was repeated twice with different batches of the three nematode isolates. Insect mortality (larvae and pupae) was determined in the same conditions as described above. At most five dead insects were randomly selected and individually dissected to confirm death by nematodes.

2.4.4. Comparative susceptibility of larvae and pupae of *Bactrocera dorsalis* to entomopathogenic nematodes

Two isolates of *H. taysearae* (Azohoue2 and Hessa1) and one of *Steinernema* sp. (Thui) were used to examine the susceptibility to EPNs of *B. dorsalis* pupae compared to that of the larvae. Third instar pupating *B. dorsalis* larvae were collected and put on sand adjusted to 10% moisture content (v/w). Pupae were collected after 24h, 48h and 72h from the same larvae batch, and pupae collected at each time were considered to be of the same age (less than one, two and three days, respectively) as we cannot know exactly when the larvae have pupated. The experiment arena consisted of a 24-well plate and nematodes were applied in the same conditions as described above at 10% moisture content with 100 IJs per pupa or larva of *B.*

dorsalis. Plates were arranged in a completely randomized block design and incubated in the dark at 28°C for 48 hours. Insect mortality (larvae and pupae) was determined in the same conditions as described above.

2.4.5. Ability of nematodes to find larva/pupa of *Bactrocera dorsalis*

H. taysearae (Azohoue2 and Hessa1) and *Steinernema* sp. (Thui) isolates were used to evaluate their ability to find *B. dorsalis* in sand substrate. Pieces of PVC tubing (diameter 4 cm) of different length (5 cm, 10 cm, 15 cm and 20 cm) were filled with sterile sand adjusted to 10% humidity. Third instar larvae of *B. dorsalis* were placed individually at one end of each piece of PVC tubing as described by Zadji et al. (2014b) and nematodes (100 IJs) were inoculated at the other end of the PVC tubing. Per combination PVC tube length × isolate, three replicates were performed, and ten PVC tubings were assigned for each replicate.

Water evaporation was controlled by closing both ends of the PVC tubings with plastic lids to maintain constant humidity during the experiment. PVC tubings were maintained vertically, with EPNs on top and the larvae at the bottom, at 28°c during 48 h after which insect mortality (larvae and pupae) was determined in the same conditions as described above. At most five dead insects were randomly selected and individually dissected to confirm death by nematodes. This assay was repeated twice with different batches of nematodes.

2.4.6. Nematode reproduction in *Bactrocera dorsalis* third instar larvae

Five dead *B. dorsalis* larvae of approximately the same size were randomly selected from the previous experiment (2.4.5) for each nematode isolate (*Heterorhabditis*: Azohoue2, Hessa1; and *Steinernema*: Thui) and placed individually on white trap to evaluate nematode reproduction potential in *B. dorsalis* larvae. Traps were incubated at 28°c. After approximately seven or five days for *Heterorhabtidis* and *Steinernema* isolates respectively, IJs were collected daily until no

nematode was observed in the white trap. The total number of nematodes produced by a single *B. dorsalis* larva was evaluated as described by Navon and Ascher (2000).

2.5. Data Analysis

Insect mortality data were corrected for control mortality according to the formula of Abbott (1925). Data obtained for all experiments were analyzed using SAS (version 16). To stabilize the variance of means, mortality percentages were arcsine transformed and subjected to a General Linear Model analysis. Student-Newman Keul's (SNK) test when $P<0.05$ was carried out to assess efficacy differences among nematode isolates. One-way analysis of variance (ANOVA) tests were conducted to determine if concentration, moisture content and migration distance had an effect on the mortality caused by EPN isolates, while two-way ANOVAs were conducted to determine whether mortality was influenced by nematode isolates, by the treatments (concentration of IJs, moisture content, host status and migration distance), or an interaction between the two. Probit regression analysis was performed in SPSS (16.0) software to calculate the LC50 of the tested nematode isolates.

3. Results

3.1. Nematode occurrence in mango orchards and identification

Two nematode isolates (KorobororouC2 and KorobororouF4) were retrieved from the 70 soil samples taken in mango orchards. This means that 2.86% of soil samples were positive. The two nematode isolates were isolated from two different mango orchards (KorobororouC2: 09°22.356'N/02°41.175'E; KorobororouF4: 09°22.287'N / 02°40.233'E) in the same village. They share 100% ITS sequence similarity with each other and with *H. taysearae*FJ477730 and 99% similarity with *H. taysearae* EF043443. Molecular identification based on the ITS regions showed that the two new nematode strains grouped with *H. taysearae* with relatively high

bootstrap value and no difference in nucleotides could not be observed with *H. taysearae* FJ477730 (Fig. 1).

Morphological (data not shown) as well as morphometrics (Supplementary material 1) information confirmed the identification of the two nematodes strains as *H. taysearae*. They share all morphological characters with *H. taysearae* previously described by Stock et al. (2009) and Zadji et al. (2013). Cross- hybridization test yielded in fertile progeny when the strains KorobororouC2 or KorobororouF4 and *H. taysearae* (KF723802, Hessa1) were crossed.

3.2. Screening of insect mortality induced by nematode isolates

All 12 tested nematode isolates infected *B. dorsalis* larvae. However, we recorded only some dead larvae (which died before pupating) and most *B. dorsalis* died as pupae (larvae which have pupated despite nematode infection). Susceptibility of *B. dorsalis* larvae was significantly different among the 12 tested nematode isolates ($F= 62.03$; $df= 11, 60$; $P<0.001$). The percentages of insect mortality varied between 7.03% and 96.09% (Table 1). The greatest insect mortality was recorded for the *H. taysearae* isolate Azohoue2 (96.09%) followed by *H. taysearae* Hessa1 (94.53%) and *H. indica* Ayogbe1 (93.75%) (Table 1). The two latter isolates were not significantly different in causing *B. dorsalis* mortality. *Steinernema* isolates Thui and Bembereke induced lower insect mortalities (69.53% and 7.03% respectively) with the latter causing the lowest mortality rate to *B. dorsalis* among all tested nematode isolates (Table 1).

3.3. Effect of nematode concentrations on *Bactrocera dorsalis* mortality

Difference in *B. dorsalis* insect mortality was significant among nematode isolates ($F= 98.89$; $df= 2, 75$; $P < 0.0001$) and among IJ concentrations ($F=31.60$; $df=4, 75$; $P< 0.0001$). However, the interaction insect mortality x IJ concentration was not significantly different ($F= 1.49$; $df =8, 75$; $P= 0.1736$). Detailed analysis showed that the three EPN isolates induced different levels of

mortality for all tested IJ concentrations (Fig. 2). The isolate *Steinernema* sp. Thui was the least virulent compared to the *H. taysearae* isolates (Azohoue2 and Hessa1) (Fig. 2).

A concentration of 100 IJs/*B. dorsalis* larva corresponding to 32 IJs / cm^2 was enough to kill at least 90% of *B. dorsalis* larvae (Fig. 2) for the *H. taysearae* isolates Azohoue2 (96.09%) and Hessa1 (94.53%) while the *Steinernema* isolate Thui could not induce the same level of mortality even when applied at high concentration (300 IJs/*B. dorsalis* larva or 95 IJs/ cm^2).

Based on the 95% confidence limits of the LC_{50} (Table 2), significant differences were observed among isolates, the highest LC_{50} was recorded with *Steinernema* sp. Thui. No significant difference was observed between the *H. taysearae* isolates, Azohoue2 and Hessa1 (Table 2).

3.4. Effect of soil moisture on the pathogenicity of EPN to *Bactrocera dorsalis*

For each nematode isolate, significant differences of *B. dorsalis* mortality were observed ($F= 3.74$; $df= 2, 75$; $P= 0.0283$). Also, at each soil moisture, IJs induced significantly different levels of *B. dorsalis* mortality ($F=42.89$; $df=4, 75$; $P<0.0001$). Furthermore, insect mortality was significantly influenced by the interactions between nematode isolates and levels of moisture content ($F=7.37$; $df= 8, 75$; $P< 0.0001$). Therefore, effect of soil moisture levels for each nematode isolate, and differences in mortality caused by nematode isolates at each soil moisture level were assessed. Significant differences in mortality caused to *B. dorsalis* by the three nematode isolates were observed only at 15% ($F=5$; $df=2, 15$; $P< 0.0217$) and 25% ($F= 16.66$; $df=2, 15$; $P < 0.0002$) moisture levels. Lower insect mortalities were recorded at 25-30% moisture levels for all tested nematode isolates (Fig. 3). All the three tested nematode isolates induced similar insect mortality at 10% and 15% moisture levels for which higher mortality rates were recorded. *H. taysearae* Azohoue2 induced higher mortality rates at 10% (99.21%) and 15% (96.76%) than at 25% (52.7%) soil moisture, while *H. taysearae* Hessa 1 and *Steinernema* sp.

Thui caused lower mortalities at 30% (70.31% and 68.75% respectively) compared to 10% (95.24% and 91.27% respectively) and 15% (96.78% and 90.32% respectively) moisture levels.

3.5. Comparative susceptibility of larvae and pupae of *Bactrocera dorsalis* to EPN

Results showed that both larvae and pupae (up to three days old) were susceptible to nematodes. Insect mortality recorded with infected larvae and 1-3 day old infected pupae revealed significant differences ($F= 400.13$; $df= 3, 60$; $P< 0.0001$) and the greater mortality level (99.21%) was recorded for infected larvae. In addition, no significant differences in insect mortality were observed among tested nematode isolates ($F=2.40$; $df= 2, 60$; $P= 0.0993$). Fully formed *B. dorsalis* pupae were less susceptible to nematodes than the third instar larvae (Fig. 4). Furthermore, susceptibility of *B. dorsalis* pupae to EPN decreased with age (Fig. 4). We recorded up to 99.21% insect mortality when nematodes were applied on *B. dorsalis* third instar larvae while insect mortality induced on 1-3 day old pupae did not exceed 23% with any of the 3 tested EPN isolates (Fig. 4).

3.6. Ability of nematodes to find larva/pupa of *Bactrocera dorsalis*

The three tested nematode isolates were capable of causing *B. dorsalis* mortality at all tested migration distances up to 20 cm except *H. taysearae* Hessa1 which induced no mortality at 20 cm. In addition, low levels of *B. dorsalis* mortality were recorded in general for all tested distances (Table 3). Results revealed that larvae/pupae mortality varied significantly with migration distance ($F=10$; $df= 3, 228$; $P<0.0001$), with greater mortality levels recorded at 5 cm (Table 3). However, no significant difference was found in *B. dorsalis* mortality according to nematode isolates used ($F= 1.42$; $df= 2, 228$; $P= 0.2431$).

3.7. Nematodes reproduction in *Bactrocera dorsalis* third instar larvae

The reproduction potential of EPNs inside third instar larvae of *B. dorsalis* varied significantly according to nematodes isolates ($F=9.26$; $df= 2, 12$; $P=0.0037$). It was possible to yield up to

59577.2 ±14307.41 IJs from larvae infested with the *Heterorhabditis* isolates (Fig. 5) which showed the greatest multiplication rate compared to the *Steinernema* one (4858.2 ±890.28 IJs per *B. dorsalis* third instars larva).

4. Discussion

In this study, we investigated the susceptibility of *B. dorsalis*, a serious mango pest in Benin, to indigenous EPNs isolates recovered from soil samples collected in mango orchards and other vegetations in Benin. EPNs are known to be more effective in their natural environment than exotic ones (Bedding, 1990). Therefore, exploring the natural occurrence of EPNs in mango orchards in northern Benin was a first step towards their application in biocontrol. Laboratory investigations to screen available isolates for effectiveness on *B. dorsalis* in variable abiotic conditions were then required before conducting field assays on a reduced number of isolates. This knowledge will help us to optimize their application in mango orchards and other environments they may be applied to. In this way, their possibilities to be introduced in the commercial market will be increased and economical losses due to *B. dorsalis* will be reduced (Gazit et al., 2000; Ma et al., 2013).

4.1. Nematode occurrence in mango orchards and identification

A prospection in several mango orchards in northern Benin revealed only 2.86% of positive samples. This percentage of positive samples is lower than reported (11.43%) in southern Benin during the rainy season by Zadji et al. (2013), but still fits the range (2% to 45%) of EPN occurrence specified by Hominick in 2002. However, the number of EPN isolates retrieved in our study from mango orchards might represent an underestimation since the prospection was done during dry season (when nematode activity is limited) over a reduced number of sites (14 mango orchards).

The identification of the two nematode isolates as *Heterorhabditis taysearae* constitutes a confirmation of the wide occurrence of that species in Benin as was reported for the first time by Zadji et al. (2013). *H. taysearae* were originally reported by Shamseldean et al. (1996) and later isolated in Mexico (Stock et al. 2009) from the Sonoran desert which shares a tropical climate with Benin.

The effective presence of EPNs in surveyed mango orchards is a promising result for any future application of EPN as this ensures that they can establish and persist in this ecological environment. Also an eventual EPN application could take into account their natural initial population in the orchard.

4.2. Screening of insect mortality induced by nematode isolates

Our laboratory experiments demonstrated the susceptibility of *B. dorsalis* larvae to all twelve tested nematode isolates. Great larval mortality (up to 96.09%) was caused by the *Heterorhabditis* strains with *H. taysearae* isolates Azohoue2 and Hessa1 being highly pathogenic to *B. dorsalis*. These results confirm earlier findings of Zadji et al. (2014) who demonstrated the same EPN isolates causing the highest mortality (98.6%) to *Macrotermes bellicosus* in citrus orchards. Furthermore, low insect mortality rates were recorded with the two tested *Steinernema* isolates with *Steinernema* sp. strain Bembereke inducing the lowest insect mortality (7.03±4.43) among all tested EPN isolates. This could be explained by the fact that IJs of *Steinernema* species penetrate an insect host only via natural openings, while *Heterorhabditis* species are equipped with an additional dorsal tooth (Griffin et al., 2005) that they use to puncture the cuticle of the insect pest to penetrate their body. *Heterorhabditis* and *Steinernema* nematodes are known to live in close association with different symbiotic bacteria (Boemare, 2002). Even though other virulence factors are involved in the death of the insect

host induced by EPNs (Ensign and Ciche, 2000; Zadji et al. 2014b), the toxicity of the associated bacterial symbiont could also be a contributing factor.

4.3. Effect of nematode concentration on *Bactrocera dorsalis* mortality

The three selected nematode isolates caused different *B. dorsalis* mortality at varying nematode concentrations with *Heterorhabditis* strains causing the highest mortality level. These results confirm those obtained above with the initial screening test where *H. taysearae* strains Azohoue2 and Hessa1 were highly pathogenic to *B. dorsalis* larvae. The highest Lc_{50} (95% confidence limit) was recorded with *Steinernema* sp. Thui isolate which confirms its lower performance in killing *B. dorsalis* larvae compared to the two *H. taysearae* isolates. For all isolates, there was no significant increase in larval mortality as nematode concentration augmented beyond 100 IJs/ larvae (or 32 IJs /cm2) meaning that a concentration of 32 IJs /cm2 was enough to obtain the optimal *B. dorsalis* mortality in our experimental conditions. This optimal EPN concentration is much lower than reported in the literature by Minas et al. (2016) and Gazit et al. (2000) who conducted similar work on other tephritid pests. For example, Minas *et* al. (2016) reported 87% mortality of *C. capitata* when a *H. baujardi* strain was applied at much higher concentration (237IJs/cm^2). In addition, Gazit *et* al. (2000) demonstrated that 100 IJs/cm^2 of *S. riobrave* could induce 82.5% of *C. capitata* mortality.

4.4. Effect of soil moisture on the pathogenicity of EPN *Bactrocera dorsalis*

We recorded significant differences in insect mortality when nematodes where applied at different moisture content (10%-30%). This means that sand moisture level influenced nematode activity in causing *B. dorsalis* mortality under our experimental conditions. Langford et al. (2014) reported significant differences in *B. tryoni* mortality when EPNs were applied at 10-25% substrate moisture. However, Gazit et al. (2000) stated that soil moisture does not affect *S. riobrave* activity in controlling *C. capitata* at larval stages. More interestingly, we observed that

nematode performance in killing *B. dorsalis* at late larval stage is reduced at high levels (25% and 30%) of soil moisture while higher mortalities were recorded at 10-15% soil moistures. This is in contrast to Langford et al. (2014) who reported low insect mortality when nematode were applied at 10% substrate moisture while higher mortality rates were observed at 25% soil moisture. Basically, nematodes are aquatic animals that require water to maintain their activity. However, some nematode species including bacterial feeding nematodes like EPNs have the ability to be active in soil even when water films are thin (Gaugler and Bilgrami, 2004). In water saturated substrate, oxygen diffusion rate may be compromised (Kaya, 1990), thus inhibiting nematode locomotion and persistence (Kung et al., 1990; Patel et al., 1997). Moreover, under conditions of high substrate moisture, nematodes are more active (Kable and May, 1968) and quickly lose their stored energy. Therefore their pathogenicity potential is reduced (Kung et al., 1991). The contrast between our results and those of Langford et al. (2014) may be due to the effect of soil moisture content on the pest itself which could die of suffocation (Hulthen and Clarke, 2006). In this respect, Shapiro et al. (2006) reported no nematode effect (though high mortality level >90%) on *Cucurlio caryae* (Pecan weevil) at 23.6% soil moisture because of the sensitivity of the latter to high moisture level. In our case, *B. dorsalis* larvae successfully complete their development at soil moisture ranging from 10 to 70% (Hou et al., 2006) which means that nematodes were the most responsible for insect mortality at that moisture level of soil.

According to Vayssières et al. (2015), *B. dorsalis* occurred in mango orchard from April to May, corresponding to the beginning of the rainy season when soil moisture is still relatively low. We therefore hypothesize based on our results that in these relatively low humidity conditions of the soil, nematode are active and control of *B. dorsalis* soil-borne stages will be enhanced, reducing then future populations of the insects in the orchard.

4.5. Comparative susceptibility of larvae and different developmental stages of *Bactrocera dorsalis* pupae to EPN.

All three EPN isolates were able to induce *B. dorsalis* larvae and pupae mortality. Late instar larvae of *B. dorsalis* were more susceptible than pupae to all nematode isolates tested. Furthermore, older pupae were less susceptible to nematodes than younger ones. We could obtain 22.8 % mortality of 1 day old pupae treated with *H. taysearae* Azohoue2 using 32 IJs / cm^2 while 5.56% mortality was recorded for 3 days old pupae treated with the same nematode isolate at the same concentration. These findings agree with earlier work of Gazit et al. (2000) who reported that *S. riobrave* could cause up to 20% mortality in young pupae of *C. capitata*. In addition, recent work of Minas et al. (2016) revealed that up 100% mortality of *C. capitata* 1 day old pupae could be achieved when applying *H. baujardi* LPP7 at higher concentration (1079 IJs/ cm2). However, this is in contrast to Langford et al. (2014), Malan and Manrakhan (2009), and Yee and Lacy (2003) who observed no pupae susceptibility of *B. tryoni*, *C. capitata/C. rosa* and *R. indifferens* respectively to EPNs. It is known that the body of pupae is much harder (due to the sclerotization) than that of the larvae, making nematode penetration through the insect cuticle (for *Heterorhabditis* species) much easier in larvae compared to pupae.

This result of positive (though sometimes low) susceptibility of *B. dorsalis* pupae to both EPN genera constitutes an opportunity for future control of this pest in field conditions as larvae which will escape parasitism by nematodes in soil could still be caught at pupal stage. We recommend further tests on older pupae (above 3 day- old pupae) to assess their susceptibility to nematodes. In addition, timing of EPNs application should be considered so as to prioritize targeting of third instar larvae.

4.6. Ability of nematodes to find larva/pupa of *Bactrocera dorsalis*

The three tested EPN isolates were able to parasitize *B. dorsalis* larvae present at 15 cm distance. *H. taysearae* Azohoue2 and *Steinernema* sp. Thui could even induce *B. dorsalis* mortality at 20 cm distance. These results confirm the cruiser type insect search strategy of the Beninese EPNs isolates described by Zadji et al. (2014b). In addition, the overall low insect mortality recorded for all tested distances could be explained by the fact that third instar larvae spend relatively short time at that stage before they switch to pupal stage which is less susceptible to EPNs than larvae as documented above. Thus, before nematodes can migrate from the inoculation point to the insect host, the latter could have pupated explaining the low mortality levels registered. In nature, third instar larvae of *B. dorsalis* leave the host fruit and migrate in soil where they pupate in the top 4cm (Hou et al., 2006). This means that they could still be reached while pupating in soil in case of future nematode application under field conditions.

4.7. Nematodes reproduction in *Bactrocera dorsalis* third instar larvae

All three tested nematode isolates were able to reproduce in *B. dorsalis* larvae. This result has great importance for nematode establishment and persistence in mango orchards as nematode populations in the orchard could be increased upon the presence of *B. dorsalis* hosts. In addition, the higher reproduction potential of *Heterorhabditis* isolates in *B. dorsalis* host compared to the *Steinernema* could be explained by differences in multiplication rate of the associated bacterial symbiont and their number released inside the host by IJs (Grewal, et al., 1997). We were able to obtain up to 59577.2 ±14307.41 IJs of *H. taysearae* produced per larval host. This value of *Heterorhabditis* isolates is considerably higher than reported by Malan and Manrakhan in 2009 when *Ceratitis rosa* larvae were infested with *H. zealandica*. A number of 6171.43 ± 814.66 IJs were counted after 19- 21 days of incubation. This wide difference may be explained by the variability in host size which relates to food availability for nematode reproduction. Indeed, the third instar larvae or pupae of *B. dorsalis* are naturally larger than

larvae or pupae of *Ceratitis rosa* as reported by Ekesi and Mohamed (2011) when those two tephritids were fed with several diets. In reality, a bigger host represented by *B. dorsalis* infected with small size IJs like *H. taysaerae* (418 µm IJ body length) should yield more progeny than a smaller Host represented by *C. rosa* infected with bigger size IJs like *H. zealendica* (685 µm IJ body length).

Overall, our results showed that the susceptibility of *B. dorsalis* to *H. taysearae* was persistent under different tested abiotic conditions which *B. dorsalis* could encounter in nature. *H. taysearae* isolates (Azohoue2 and Hessa1) therefore represent potential biological agents that may be used in the control of *B. dorsalis* in mango orchards. The concentration of 100 IJs / larva applied at 10-15% soil moisture showed optimal results in laboratory tests. Fields trials are now required to test the effectiveness of *B. dorsalis* to these *Heterorhabditis* isolates under natural environmental conditions.

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Figure captions:

Fig. 1. Phylogenetic relationships based on Neighbor Joining clustering of *Heterorhabditis* ITS sequences showing the position of both nematode strains retrieved from mango orchards in northern Benin (shown in bold). H: *Heterorhabditis*, C: *Caenorhabditis,* S: *Steinernema*. Numbers at the nodes indicate bootstrap value (1000 replicates) and numbers after species in parentheses represent nucleotide differences between ITS sequences of described species and the newly isolated *Heterorhabditis* strains from mango orchards.

Fig. 2. Effect of different concentrations (20, 50, 100, 200, and 300 IJs/*B. dorsalis* larva) of two isolates of *H. taysearae* (Azohoue2 and Hessa1) and one of *Steinernema* sp. (Thui) on *B. dorsalis* mortality (% ±SEM). Vertical bars are standard error of the means. Bars with the same uppercase letter stand for non-significant differences existing among nematode isolates causing *B. dorsalis* mortality at the same concentration level. Bars with the same lowercase letter stand for non-significant differences existing among nematode concentrations causing *B. dorsalis* mortality for the same nematode isolate (SNK's test at $P<0.05$).

Fig. 3. Effect of moisture content (10%; 15%; 20%; 25% and 30%) of the substrate (sterile sand) on *B. dorsalis* mortality (% ±SEM) exposed to the three tested nematode isolates (Azohoue2; Hessa1 and Thui). Vertical bars are standard error of the means. Bars with the same uppercase letters stand for non-significant differences existing among levels of moisture content for the same nematode isolate. Bars with the same lowercase letters stand for non-significant differences among nematode isolates for the same level of moisture content (SNK's test at $P<0.05$).

Fig. 4. Comparison of susceptibility of *B. dorsalis* pupae and third instar larvae (% ± SEM) to three nematodes isolates. Vertical bars are standard error of the means. Bars with different letters stand for significant differences among insect developmental stages (SNK's test at $P<0.05$).

Fig. 5. Reproduction potential of three nematode isolates in third instar larvae of *B. dorsalis*. Vertical bars are standard errors of the means. Bars with the same letters stand for non-significant differences of EPN reproduction among tested nematode isolates (SNK's test at $P<0.05$).

Fig. 1

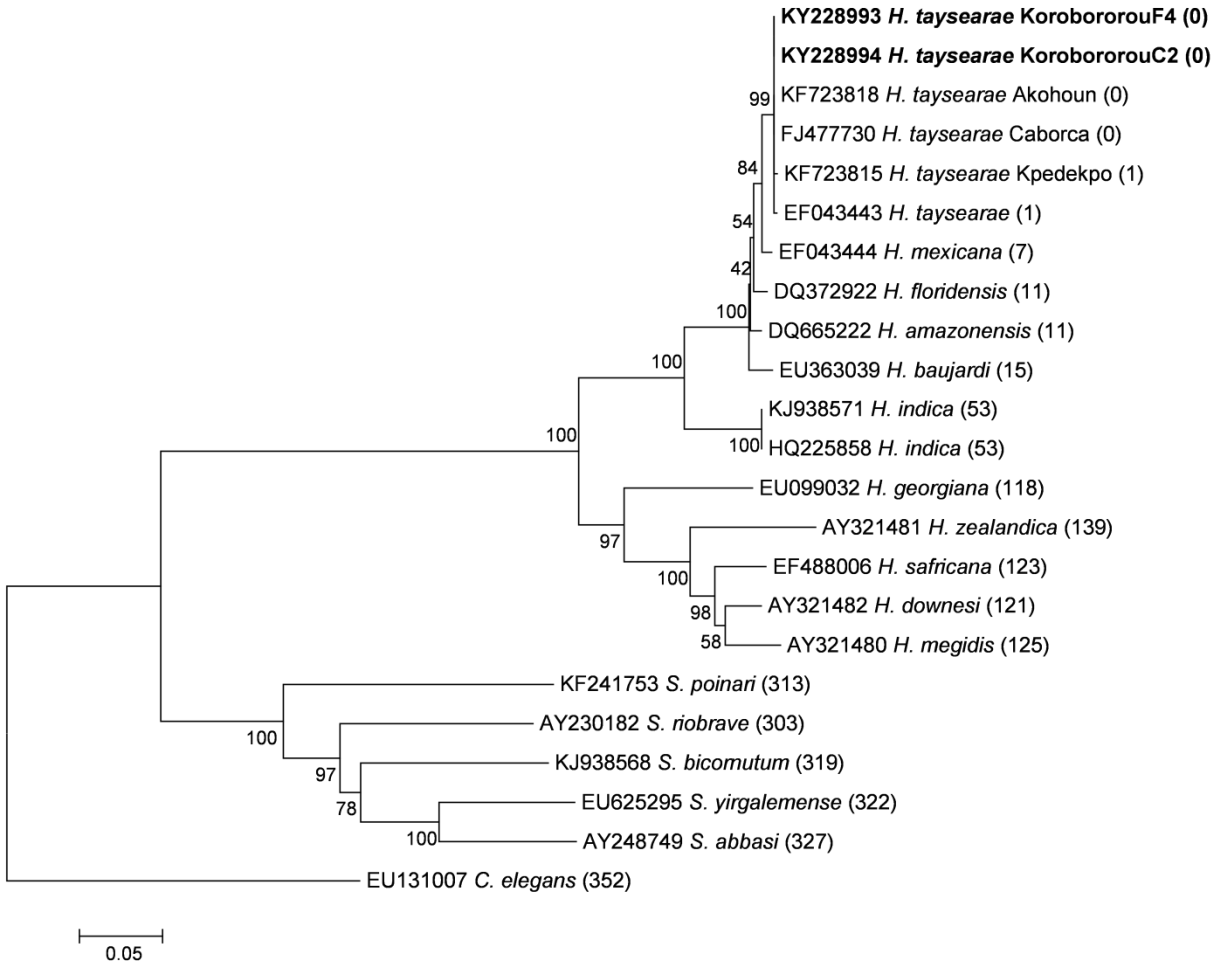
KY228993 H. taysearae KoroborourouF4 (0)
KY228994 H. taysearae KoroborourouC2 (0)
KF723818 H. taysearae Akohoun (0)
FJ477730 H. taysearae Caborca (0)
KF723815 H. taysearae Kpedekpo (1)
EF043443 H. taysearae (1)
EF043444 H. mexicana (7)
DQ372922 H. floridensis (11)
DQ665222 H. amazonensis (11)
EU363039 H. baujardi (15)
KJ938571 H. indica (53)
HQ225858 H. indica (53)
EU099032 H. georgiana (118)
AY321481 H. zealandica (139)
EF488006 H. safricana (123)
AY321482 H. downesi (121)
AY321480 H. megidis (125)
KF241753 S. poinari (313)
AY230182 S. riobrave (303)
KJ938568 S. bicornutum (319)
EU625295 S. yirgalemense (322)
AY248749 S. abbasi (327)
EU131007 C. elegans (352)
99
84
54
42
100
100
100
100
97
100
98
58
100
97
78
100
0.05

Fig. 2

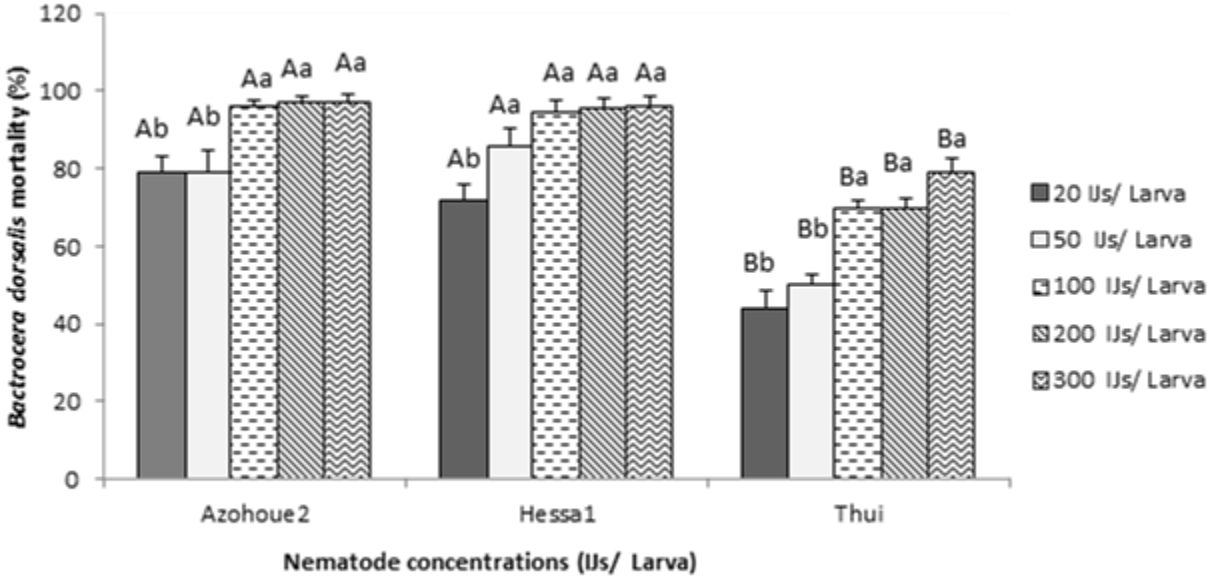
120
100
80
60
40
20
0
Bactrocera dorsalis mortality (%)
Ab
Ab
Aa
Aa
Aa
Ab
Aa
Aa
Aa
Aa
Bb
Bb
Ba
Ba
Ba
Azohoue2
Hessa1
Thui
Nematode concentrations (IJs/ Larva)
20 IJs/ Larva
50 IJs/ Larva
100 IJs/ Larva
200 IJs/ Larva
300 IJs/ Larva

Fig. 3

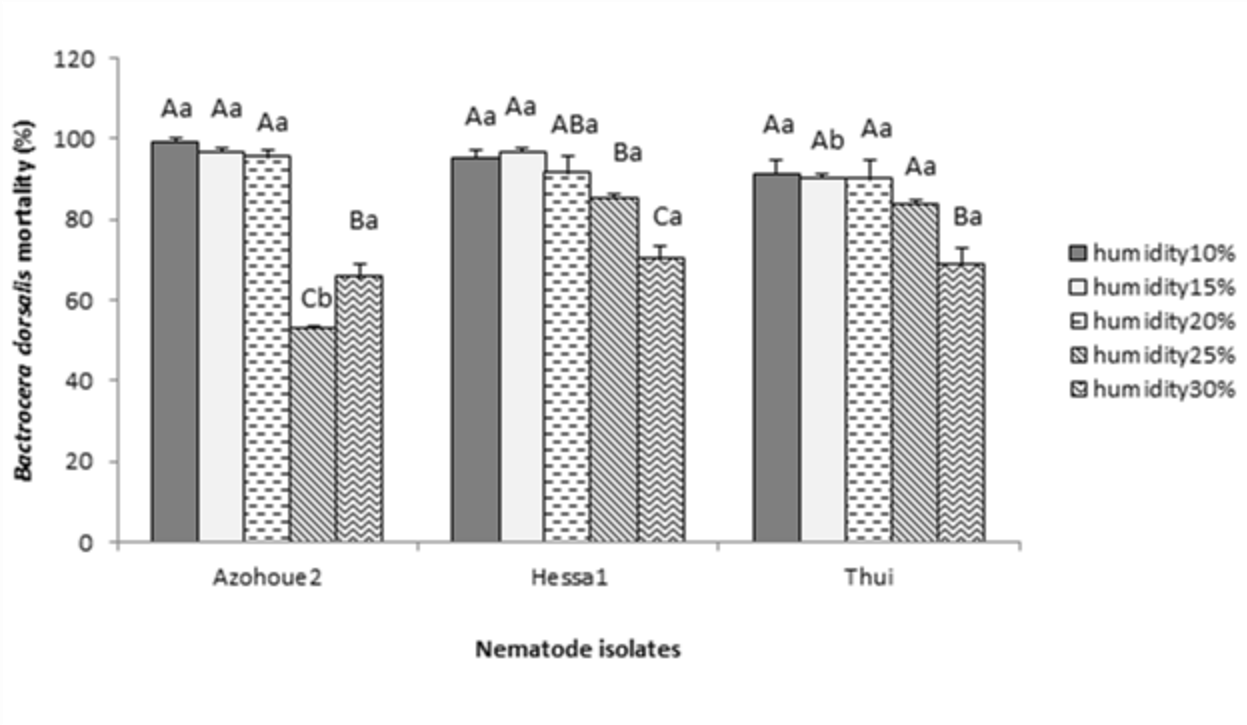
120
100
80
60
40
20
0
Bactrocera dorsalis mortality (%)
Aa Aa Aa Cb Ba
Aa Aa ABa Ba Ca
Aa Ab Aa Aa Ba
Azohoue2
Hessa1
Thui
Nematode isolates
humidity10%
humidity15%
humidity20%
humidity25%
humidity30%

Fig. 4

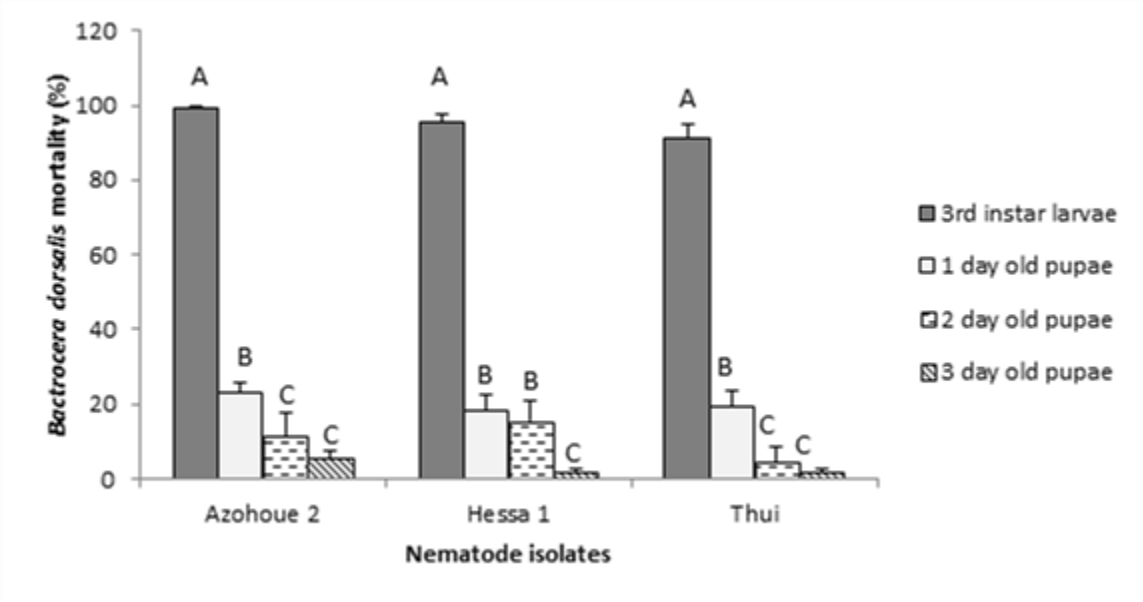
120
100
80
60
40
20
0
Bactrocera dorsalis mortality (%)
A
B
C
C
A
B
B
C
A
B
C
C
Azohoue 2
Hessa 1
Thui
Nematode isolates
3rd instar larvae
1 day old pupae
2 day old pupae
3 day old pupae

Fig. 5

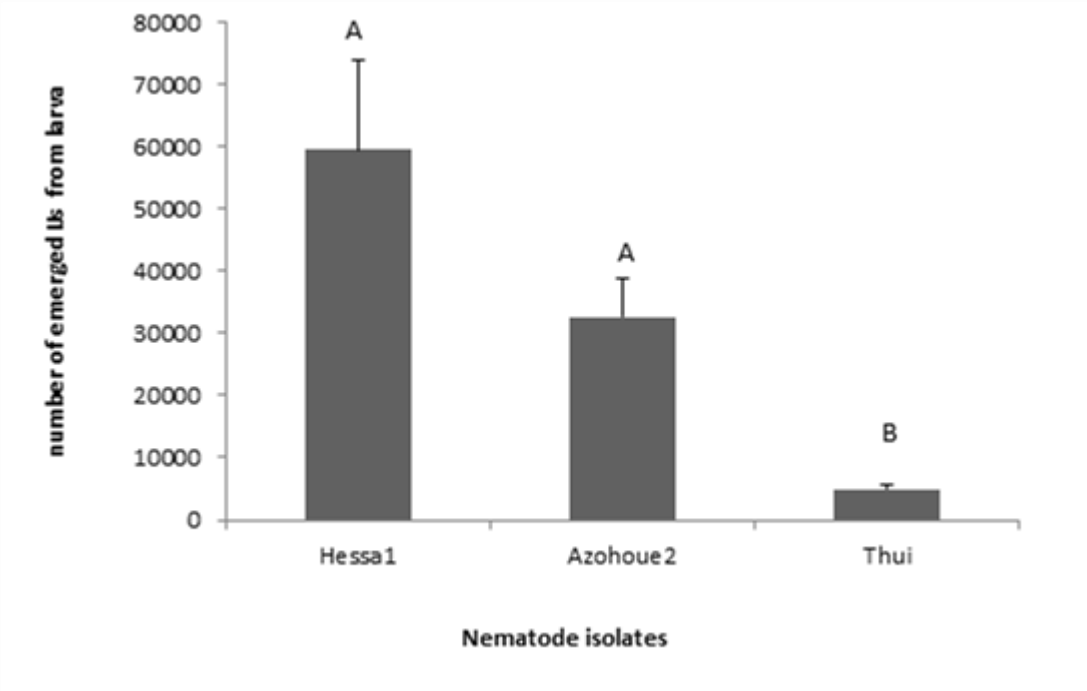
number of emerged ls from larva
80000
70000
60000
50000
40000
30000
20000
10000
0
A
A
B
Hessa1
Azohoue2
Thui
Nematode isolates

Table 1

Characteristics of the 12 studied EPN isolates from Benin and mortality (%±SEM) caused to *B. dorsalis*

Sampling number	Nematode species	ITS accession number	Vegetation	Origin in Benin	References	% Mortality of *B. dorsalis* (±SEM)*
32b	*H. taysearae*	KF723809	Mandarin	Azohoue2	Zadji et al., 2013	96.09±1.44 a
9a	*H. taysearae*	KF723802	Lemon	Hessa1	Zadji et al., 2013	94.53±2.82 ab
51a	*H. indica*	KF723816	Mango	Ayogbe1	Zadji et al., 2013	93.75±2.32 ab
83a	*H. taysearae*	KF723828	Palm	Ze3	Zadji et al., 2013	90.62±3.42 abc
59a	*H. taysearae*	KF723818	Teak	Akohoun	Zadji et al., 2013	85.93±1.71 abcd
9d	*H. taysearae*	KF723803	Lemon	Hessa2	Zadji et al., 2013	82.81±0.99 bdc
44a	*H. taysearae*	KF723813	Orange	Kemondji	Zadji et al., 2013	76.56±4.01 de
F4**	*H. taysearae*	KY228993	Mango	Korobororou F4	This study	79.69±4.47 dec
168d	*Steinernema* sp.	KY228996	Eucalyptus	Thui	Unpublished	69.53±2.00 ef
118c	*H. taysearae*	KY228995	Cashew	Gouka	Unpublished	64.06±3.12 f
C2**	*H. taysearae*	KY228994	Mango	Korobororou C2	This study	51.56±4.63 g
157c	*Steinernema* sp.	KY228997	Gallery forest	Bembereke	Unpublished	7.03±4.43 h

SEM: Standard Error of the Mean.

* Means with the same letter are not significantly different. ** EPN isolates retrieved from soil sampled in mango orchards during January-February 2015 survey.

Table 2

Comparison of lethal concentrations (LC_{50}) of two *Heterorhabditis* isolates (Azohoue2 and Hessa1) and one *Steinernema* isolate (Thui) against *B. dorsalis* larvae.

Nematode Isolates	**Origin of Nematode isolates**	**Probit equation[a]**	**χ^2**	**LC_{50}[b]**	**95% CL[c]**
H. taysearae	Azohoue2	Y= - 0.710+1.071C	8.113	4.603 b	0-18.785
H. taysearae	Hessa1	Y= - 0.803+1.097C	1.642	5.396 b	1.488-10.511
***Steinernema* sp.**	Thui	Y= - 1.253+0.810C	2.973	35.205a	19.503-50.820

[a] General responses of insect mortality (Y) as a function of nematode concentration (C).

[b] Nematode concentration (number of IJs per *B. dorsalis* larva) required for killing 50% of treated larvae; LC_{50} values followed by the same letter are not significantly different, based on non-overlapping 95% CL.

[c] 95% confidence limits (CL) for the LC_{50}.

Table 3

Mortality of *B. dorsalis* (Means ± SEM) caused by different nematodes isolates at 5, 10, 15 and 20 cm depth (distance between insect and nematode inoculation point).

Nematode isolates	Mortality (% ± SEM)			
	5 cm distance	10 cm distance	15 cm distance	20 cm distance
H. taysearae Azohoue2	45 ± 11.41 Aa	20 ± 9.17 Ba	5 ±5.00 Ba	5± 5.00 Ba
H. taysearae Hessa1	40 ± 11.24 Aa	10 ± 6.88 Ba	5 ±5.00 Ba	0.00 Ba
Steinernema sp. Thui	15 ± 8.19 Aa	10 ± 6.88 Aa	10 ± 6.88 Aa	5 ± 5.00 Aa

SEM: Standard Error of the Mean.

Means (% ± SEM) with the same uppercase letter are not significantly different for the same nematode isolate. Means with the same lowercase letters stand for non-significant differences among nematode isolates for the same migration distance (SNK's test; $P< 0.05$).

Supplementary material 1

Comparative table of morphometrics of infective juveniles of *H. taysearae* isolates KoroborororouC2 and KoroborororouF4 from Benin and described *H. taysearae* species (in µm, ± standard error and range in parenthesis).

Characters	***H. taysearae*** **(this study) Koroborororou C2 isolate**	***H. taysearae*** **(this study) Koroborororou F4 isolate**	***H. taysearae*** **(Stock et al., 2009) Caborca isolate**	***H. taysearae*** **(Shamseldean et al., 1996)**
n	20	20	20	30
L	**570.39 ± 38.36 (512-615)**	**501.67 ± 35 (459-580)**	**557 ± 28 (495-570)**	**418 ± 38 (332-499)**
a	24,76 ± 2.35 (19-28)	21.60 ± 2.43 (17-27)	23 ± 1.5 (19-26)	21 ± 2.2 (18-27)
b	4.59 ± 0.31 (4-5)	4.30 ± 0.33 (3-5)	4.8 ± 0.4 (4.4-5.4)	3.8 ± 0.2 (3.4-4.2)
c	**9.48 ± 1.07 (7-10)**	**8.66 ± 0.7 (7-9)**	**5.5 ± 1.0 (4.0-6.5)**	**7.7 ± 0.7 (6.5-8.7)**
MBD	23.18 ± 2.25 (20-27)	23.41 ± 2.4 (20-29)	25.5 ± 4 (19-32)	20 ± 1.9 (17-23)
EP	**102.95 ± 7 (91-114)**	**102.1 ± 8.96 (82-115)**	**99 ± 4.5 (97-116)**	**90 ± 9.1 (74-113)**
NR	**93.24 ± 5.19 (87-108)**	**91.35 ± 7.74 (79-105)**	**93 ± 4 (87-98)**	**64 ± 6.8 (58-87)**
ES	**124.33 ± 6.63 (111-144)**	**117.13 ± 11.59 (95-136)**	**119 ± 7 (110-131)**	**110 ± 8.4 (96-130)**
T	**60.69 ± 6.5 (49-77)**	**58.42 ± 7.52 (48-70)**	**105 ± 7 (91-125)**	**55 ± 6.6 (44-70)**
ABD	11.77 ± 1.47 (9-16)	12.89 ± 1.7 (10-16)	16 ± 2 (13-16)	-
D%	**82.85 ± 4.73 (73 - 89)**	**87.32 ± 3.87 (81-97)**	**90 ± 8.5 (78-110)**	**82 ± 6 (71-96)**
E%	**171.51 ± 22.07 (135-212)**	**174.75 ± 17.05 (137-205)**	**99 ± 8 (81-111)**	**180 ± 27 (110-230)**

L= Body length, MBD= Maximum Body Diameter, EP= distance from anterior end to secretory-excretory pore, ES= pharynx length, NR = Nerve Ring, T=tail length, ABD= Anal Body Diameter, a= L/MBD, b= L/T, c= L/T, D%= (EP/ES) x 100, E%= (EP/T) x 100

Highlights

- Entomopathogenic nematodes (EPNs) from Benin cause mortality in *Bactrocera dorsalis* late instar larvae and pupae.
- Nematode concentrations as well as substrate humidity influence the susceptibility of *Bactrocera dorsalis* to EPNs.
- EPNs from Benin are able to reproduce in *B. dorsalis* larvae and pupae