# RESEARCH/INVESTIGACIÓN

## SURVEY OF ENTOMOPATHOGENIC NEMATODES IN VARIOUS LANDSCAPE SYSTEMS ON OAHU, HAWAII, AND THEIR PATHOGENICITY AGAINST COCONUT RHINOCEROS BEETLE (COLEOPTERA: SCARABAEIDAE)

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## ABSTRACT

Manandhar, R., J. Li, and Z. Cheng. Survey of entomopathogenic nematodes in various landscape systems on Oahu, Hawaii, and their pathogenicity against coconut rhinoceros beetle (Coleoptera: Scarabaeidae). Nematropica 50:36-44.

The use of locally available biological control agents (e.g., entomopathogenic nematodes, EPNs) can be an important tool in integrated pest management (IPM) programs of newly established invasive pests, such as coconut rhinoceros beetle (CRB), *Oryctes rhinoceros* (L.). With this aim, we surveyed EPNs from 12 landscape sites that included four public/recreational areas, six botanical gardens, and two forest reserves on the island of Oahu, HI. Overall, 35 (83.3%) of the total samples were found positive for EPNs and out of those samples, 20 (57.1%) samples yielded virulent strains. EPNs recovered from the surveyed sites were primarily composed of *Heterorhabditis* spp. Some *Heterorhabditis* spp. virulent strains were tested for pathogenicity against first instar CRB larvae. Although our study did not show significant effects of *Heterorhabditis* spp. strains against CRB larvae, some strains inflicted mortality of first instar larvae after 2 days as well as a week after exposure. Our findings indicated that the locally available strains of *Heterorhabditis* spp. could potentially be a useful biological control agent, in an IPM program, to control first instar CRB larvae. However, further research is needed to test virulent commercial strains of Heterorhabditid and Steinernematid against CRB larvae. The results of such studies will uncover if certain EPN strains could be incorporated as a part of CRB IPM program.

*Key words:* Biological control, *Heterorhabditis* spp., virulent strains, mortality

## RESUMEN

Manandhar, R., J. Li, y Z. Cheng. Estudio de nematodos entomopatógenos en varios sistemas de paisaje en Oahu, Hawaii, y su patogenicidad contra el escarabajo rinoceronte del coco (Coleoptera: Scarabaeidae). Nematropica 50:36-44.

El uso de agentes de control biológico disponibles localmente (e.g., nematodos entomopatógenos, NEPs) pueden ser una importante herramienta en los programas de manejo integrado de plagas (MIP), especialmente las plagas invasivas recién establecidas, como el escarabajo rinoceronte del coco (ERC), *Oryctes rhinoceros* (L.). Con este objetivo, se estudiaron NEPs de 12 sitios en paisajes que incluyeron

cuatro áreas públicas/recreacionales, seis jardines botánicos, y dos reservas forestales en la isla de Oahu, HI. En general, 35 (83,3%) del total de las muestras dieron positivas para NEPs y de estas muestras, 20 (57,1%) resultaron en cepas virulentas. Los NEPs recuperados de los sitios estudiados estaban compuestas principalmente por *Heterorhabditis* spp. Algunas cepas virulentas de *Heterorhabditis* spp. se analizaron para determinar su patogenicidad contra larvas en primer estado del ERC. Aunque nuestro estudio no mostró efectos significativos de las cepas de *Heterorhabditis* spp. contra larvas de ERC, algunas cepas infligieron mortalidad del primer estado larval luego de dos días y una semana después de la exposición. Nuestros hallazgos indicaron que las cepas de *Heterorhabditis* spp. disponibles localmente podrían ser un agente de control biológico útil en un programa MIP para manejar el primer estado larval de ERC. Sin embargo, se necesita más investigación para probar cepas virulentas comerciales de Heterorhabditid y Steinernematid contra las larvas de ERC. Los resultados de estos estudios determinarán si algunas cepas de NEP pueden incorporarse como parte del MIP de ERC.

*Palabras clave:* Control biológico, *Heterorhabditis* spp., cepas virulentas, mortalidad

## INTRODUCTION

Among the diversity of insect parasitic nematodes, entomopathogenic nematodes (EPNs) are distinct in killing insect hosts rapidly with the aid of their bacterial partners (Dillman *et al*., 2012). EPNs of the families, Heterorhabditidae and Steinernematidae, are the most common and have been isolated from soil-inhabiting insects throughout the world (Kaya and Gaugler, 1993; Poinar and Grewal, 2012). EPNs occur naturally in the soil and are widely distributed in managed and natural ecosystems worldwide. The naturally occurring EPNs are typically isolated from the soil using the *Galleria* bait method (Akhurst and Brooks, 1984; Hara *et al*., 1991). Surveys for EPNs have been conducted in natural and managed ecosystems worldwide (Akhurst and Bedding, 1986; Liu and Berry, 1995), including Hawaii, to unravel their occurrence and distribution patterns (Hara *et al*., 1991; Myers *et al*., 2015). The existence of EPNs in Hawaii (Hara *et al*., 1991; Myers *et al*., 2015) and the state's quarantine enforcement concerning importation of exotic organisms prompted a call for the exploration of locally occurring EPNs that could be used in biological control programs. In addition, locally occurring EPNs are better adapted to local soil and environmental conditions (Campos-Herrera *et al*., 2013; Salame and Glazer, 2015); and are expected to play a greater role in suppressing ground-dwelling insect pests. Therefore, we aimed to survey locally occurring EPNs, specifically in various landscape systems on Oahu, HI, because: 1) their potential hosts (ground-dwelling insects) may be more diverse and abundant compared to agricultural systems, 2) landscape systems, especially urban landscapes, have not been focused on in previous EPN surveys in Hawaii, and 3) the potential of EPNs in controlling the coconut rhinoceros beetle, an invasive pest of urban landscapes that recently established on Oahu, HI, has not been evaluated.

The coconut rhinoceros beetle (CRB), *Oryctes rhinoceros* L. (Coleoptera: Scarabaeidae) is a serious pest of coconut trees, *Cocos nucifera* L. (Arecaceae) and other palm species. This beetle is native to Southeast Asia and has spread through the South Pacific. In Hawaii, the first adult was detected in December 2013 on the island of Oahu. Since then, an incipient CRB population exists on the island and is currently the target of a large, multiagency eradication program (Watanabe and Melzer, 2017). Adult beetles bore into the crown of palms and feed on sap and can kill palms if the infestation is severe. Although CRB damage does not always result in coconut tree mortality, the characteristic V-cut damage to palm fronds can adversely affect the aesthetic value (Hinckley, 1973; Bedford, 1980). In contrast, CRB larvae cause no damage, and reside in cryptic breeding sites as they feed on decaying materials, which mainly include dead standing and fallen coconut trees (Moore *et al*., 2017). Since tourism is the largest contributor to Hawaii's economy (Hawaii Tourism Authority, 2017), the attraction of iconic sites on the island needs to be preserved through safeguarding palm trees. Typically, CRB populations are controlled by implementing integrated pest management (IPM) techniques with a combination of biocontrol agents, chemical control, pheromone traps, and breeding site

removal (Bedford, 1980; 2013). Among the biocontrol agents, the use of entomopathogenic fungi and baculoviruses has shown some potential to control CRB populations (Bedford, 2013), but the effectiveness of EPNs against CRB is not known. The use of EPNs has been found effective against some Scarabaeidae larvae (Gyawaly *et al*., 2016) and can be an important component of IPM (Kaya and Gaugler, 1993). Therefore, an interest was raised to determine whether the use of locally occurring EPNs would be an effective management tool for controlling CRB. The main objective of this study was to identify locally available virulent EPNs and test these strains for pathogenicity against CRB larvae with the potential to incorporate into a CRB IPM program.

## MATERIALS AND METHODS

*Survey of EPNs*

A total of 12 landscape sites on Oahu were included in the survey of EPNs. Among them, four were public/recreational sites [University of Hawaii at Manoa (UHM) Campus, Ala Moana Beach Park, Iroquois Point, and Ala Wai Golf Course], six were botanical gardens [Harold H. Lyon Arboretum, Queen Liliuokalani Botanical Garden, Hoomaluhia Botanical Garden, Koko Crater Botanical Garden, Wahiawa Botanical Garden, Waimea Botanical Garden], and two were forest reserves [Mokuleia Forest Reserve and Makua Keaau Forest Reserve] (Table 1, Fig. 1). A stratified random sampling technique was used to survey these sites from February 2016 to January 2017. In each site, the survey area was stratified into 2-4 zones and 4 random soil samples were collected from each zone. Soil samples were collected from the grassy areas around various landscape plants such as Chinese and Weeping banyans (*Ficus* spp.), Monkey pod tree (*Samanea saman*), Coconut palm (*C. nucifera*), and Wiliwili tree (*Erythrina sandwicensis*). In a few cases when these landscape plants were not available, samples were taken from other palms and *Hibiscus* spp. (Wahiawa Botanical Garden), and *Plumeria* and Cactus collections (Koko Crater Botanical Garden). Samples were taken to a depth of 10 cm, using a ground shark shovel and placed in plastic Ziploc bags. In the laboratory, soil samples from each zone were thoroughly mixed by eliminating rocks, clods, and plant parts to make a composite sample. One-fourth of this sample was moistened to its field capacity by adding water prior to EPN isolation. In addition, a portion of each composite sample was used to determine soil texture by feel method as described in the Natural Resources Conservation Service's Guide (Natural Resources Conservation Services, n.d.).

Due to the strict regulation of importation of exotic organisms by the Hawaii Department of Agriculture (HDOA), the *Galleria* bait method was not used for EPN isolation. Instead, EPNs were isolated by using an alternative insect, mealworm *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) bait method (Bisel *et al*., 2016). Twelve 3$^{rd}$ instar mealworm larvae were placed in a 1-L capacity plastic container, and the larvae were covered by a processed composite soil sample. This plastic container was filled with the soil sample up to two-thirds of its capacity and secured with a screw top lid with a hole at the center. The position of the container was inverted each day so that the larvae would encounter a majority of the soil particles. The mealworm larvae were examined after the third day of baiting and baiting continued for five consecutive days. Any dead larvae encountered were taken out and rinsed in 10% bleach solution followed by tap water twice. The rinsed larvae were then placed in modified White traps for collecting infective juveniles (White, 1927; Hara *et al*., 1991). The White trap consisted of an inverted Petri dish (60-mm diam.) cover within a larger Petri dish (100-mm diam. by 25 mm height). The water suspension on traps was examined after 10 days using an inverted microscope to confirm the presence of infective juveniles (IJs). The suspension containing EPNs was collected after 14 days in a 50-ml centrifuge tube and stored in an incubator at 15˚C. EPNs collected from each composite sample represented an EPN-positive strain from a survey site. Further, each EPN strain was inoculated on mealworms to maintain and confirm virulence on monthly intervals. The virulent strains of EPNs were identified based on the ability of a strain to infect mealworm larvae and yield IJs in three successive inoculations.

Table 1. List of survey sites for entomopathogenic nematodes (EPNs) included in each landscape system on the island of Oahu, Hawaii. The table shows the number of soil samples collected, number of soil samples that yielded EPNs, number of virulent EPN strains (*Heterorhabditis* spp.) yielded from EPN-positive samples, and the code for each virulent strain (February 2016 to January 2017).

| Sites | Location | Altitude | Soil texture | No. samples collected | No. of samples positive to EPNs | No. of virulent strains | Virulent strains |
|---|---|---|---|---|---|---|---|
| *Public/recreational sites* | | | | | | | |
| UH Manoa Campus | Honolulu | 27 m | Sandy clay loam | 2 | 2 | 2 | UH-1, UH-2 |
| Ala Moana Beach Park | Honolulu | 4 m | Sandy clay loam | 4 | 4 | 2 | AM-1, AM-4 |
| Iroquois Point | Ewa | 4 m | Sandy clay | 4 | 3 | 1 | IQ-4 |
| Ala Wai Golf Course | Honolulu | 3 m | Clay loam | 4 | 3 | 0 | - |
| *Botanical gardens* | | | | | | | |
| Harold L. Lyon Arboretum | Honolulu | 158 m | Clay loam | 4 | 4 | 3 | Lyon-1, Lyon-2, Lyon-4 |
| Queen Liliuokalani Botanical Garden | Honolulu | 14 m | Clay loam | 2 | 2 | 2 | Lil-1, Lil-2 |
| Hoomaluhia Botanical Garden | Kaneohe | 49 m | Clay loam | 4 | 4 | 3 | Hoo-2, Hoo-3, Hoo-4 |
| Koko Crater Botanical Garden | Hawaii Kai | 110 m | Sandy clay loam | 4 | 4 | 3 | Koko-2, Koko-3, Koko-4 |
| Wahiawa Botanical Garden | Wahiawa | 296 m | Clay loam | 2 | 2 | 2 | Wah-1, Wah-2 |
| Waimea Botanical Garden | Haleiwa | 123 m | Clay loam | 4 | 4 | 0 | - |
| *Forest reserves* | | | | | | | |
| Mokulei Forest Reserve | Waialua | 381 m | Sandy clay loam | 4 | 2 | 1 | Moku-3 |
| Makua Keaau Forest Reserve | Waianae | 377 m | Sandy clay loam | 4 | 1 | 1 | Keaau-3 |

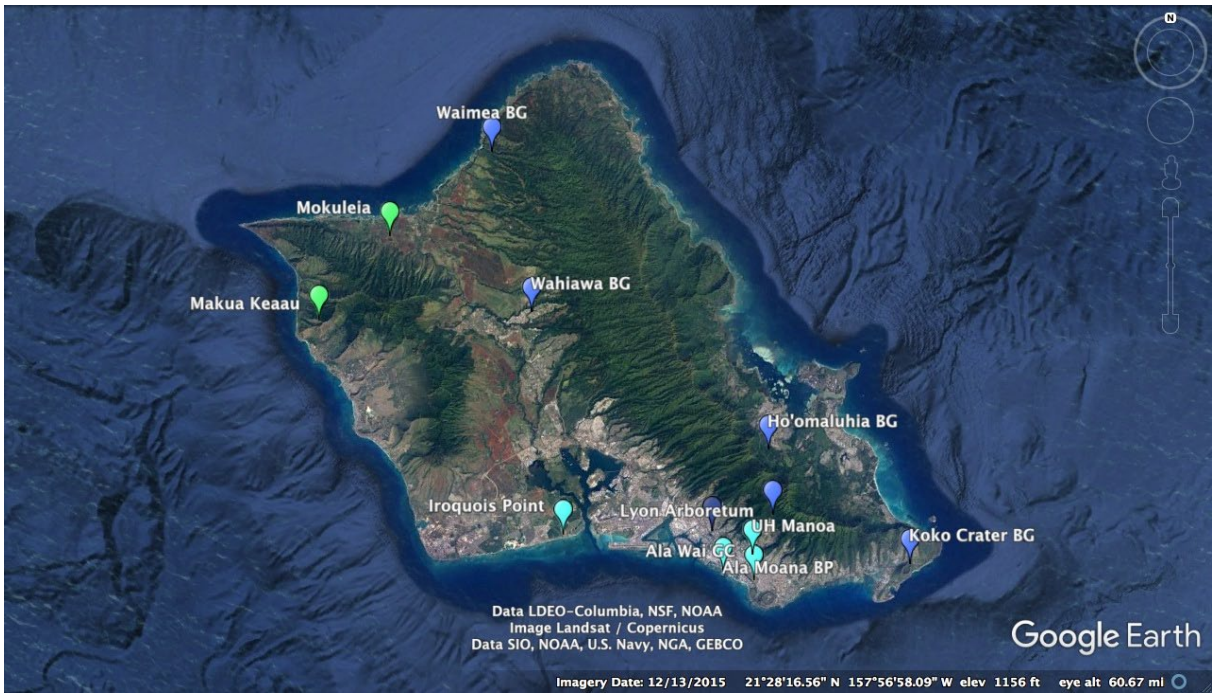


Figure 1. Map of survey sites for entomopathogenic nematodes on the island of Oahu in this study. Blue icon represents botanical gardens (BG), light blue icon represents public/recreational sites (BP = beach park, GC = golf course), and green icon represents forest reserves.

*Pathogenicity of EPNs on CRB larvae*

As a part of CRB eradication program, the original CRB-rearing facility established at the Plant Quarantine Division, Hawaii Department of Agriculture (HDOA), was in the process of being moved to the Arthropod Containment Facility (ACF), Department of Plant and Environmental Protection Sciences (PEPS) at UHM. Due to this reason, the production of CRB larvae was slow and limited, and it was necessary to conduct a series of small trials using available numbers of CRB larvae each time. Preliminary experiments were conducted to determine the optimum rate of EPNs to cause larval mortality, whether the larvae should be treated in a group or individually, and the susceptible stage of larvae to EPNs. These experiments showed that the higher rate of IJs (1,000 IJs/CRB larvae) was needed to cause larval mortality, larvae in a group showed cannibalistic behavior, and the first instar larvae were more susceptible than second instar larvae. Based on these results, we developed a bioassay procedure to test the pathogenicity of virulent strains of EPNs against first instar larvae. For this, a single first instar larva in a 96-ml capacity plastic Solo cup was used as an experimental unit in the laboratory bioassay trials. EPNs were applied at 1,000 IJs/larva as a treatment and 0 IJs as a control. The larva was then covered with approximately 10 g of moist compost mix (50:50 = Menehune soil mix: Coco coir). The Solo cups were securely covered with a lid containing several small holes.

A total of four trials were conducted to test the pathogenicity of EPN strains on CRB first instar larvae monthly from February-May 2017. In each trial, EPN strains yielding greater numbers of IJs during the routine laboratory rearing process were chosen as the treatments. Therefore, the EPN strains used were not always the same among the trials. The number of strains ranged from six to nine, based on the availability of larvae at the time of a trial. The number of experimental units was maintained at 10 each for the treatments and the control, except in the last trial (May) when the experimental units were 7 and 8 for 2 treatments (Wah-2 and Lyon-2). Each trial was considered as a replicate; however, the variable numbers of strains in each trial produced an unbalanced completely randomized block design.

Observations on larval mortality were first assessed after 2 days of exposure and continued daily for 5 consecutive days. The dead CRB larvae showing typical EPN infection symptoms, if any, were placed in the White trap to confirm that mortality was caused by EPNs. This experiment was conducted in the ACF, Department of PEPS, and within this facility the containers were placed in a incubator at 25°C with the source of light turned off.

*Data analysis*

The percentages of EPN-positive strains and virulent EPN strains were calculated by landscape types. The percentage of EPN-positive strains was calculated from the total number of samples collected, whereas the percentage of virulent EPN strains was calculated from the number of EPN positive strains. The percentage mortality of CRB larvae, after two days and after a week of exposure, was calculated for each trial. EPN strains that were replicated at least twice (2 trials) were accommodated in the final data analysis. There was a total of nine EPN strains compared with the control. The percentage mortality of CRB larvae was analyzed using a general linear model in analysis of variance (ANOVA) (PROC GLM, SAS Institute). A statistical model included "treatment" as a fixed factor and "trial" as a random factor. The percentage data for CRB mortality were arcsine [√(percentage/100)] transformed to stabilize variances. Means were separated using Tukey's HSD test at 0.05% level of significance whenever applicable. Reported means are from non-transformed data.

## RESULTS

*Survey of EPNs*

The public/recreational sites were located close to the coastal area, except UHM campus, and periodically irrigated. The botanical gardens were located mostly in the inland and are generally wet through various sources of water at the site in the form of falls, streams, and ponds, except Koko Crater Botanical Garden, which is dry. The forest reserve sites were located close to the coastal areas, but comparatively at a higher altitude and generally composed of dry soil, cinders, and rocks. The soil texture was similar among the sites with varying levels of sand and clay that ranged from sandy-clay to clay-loam.

In total, 42 soil samples were collected from 12 different sites, and EPNs were isolated from all the sites. The number of samples collected, EPN positive samples, and the number and code of the virulent strains are listed in the Table 2. Overall 35 (83.3%) of the total samples were found positive for EPNs and out of those samples, 20 (57.1%) samples yielded virulent strains. Among the different categories of sites, 20 (100%) of soil samples from botanical garden followed by 12 (85.7%) from public/recreational sites, and 3 (37.5%) from the forest reserves were found positive for EPNs. Out of these positive samples, 13 (65.5%) from botanical gardens, 5 (41.7%)

Table 2. List of landscape types including associated plants in each landscape from a survey of entomopathogenic nematodes (EPNs) on the island of Oahu, Hawaii (February 2016 to January 2017). The total number of soil samples collected, number of soil samples that yielded EPNs, and number of virulent EPN strains (*Heterorhabditis* spp.) yielded from EPN-positive samples were determined.

| Landscape ecosystems/ types | Associated landscape plants | No. of samples collected | No. of positive samples (%) | No. of virulent strains (%) |
|---|---|---|---|---|
| Public/recreational | Chinese and weeping banyans, Monkey pod tree, Coconut palm | 14 | 12 (85.7) | 5 (41.7) |
| Botanical gardens | Wiliwili tree, ornamental collections (Palms, *Hibiscus*, *Plumeria*, Cactus etc.) | 20 | 20 (100.0) | 13 (65.0) |
| Forest reserves | Wiliwili tree | 8 | 3 (37.5) | 2 (66.7) |
| Totals | | 42 | 35 (83.3) | 20 (57.1) |

from public/recreational sites, and 2 (66.7%) from forest reserves yielded virulent strains (Table 2). Based on the morphological features of EPNs, the strains consisted of EPNs in the Heterorhabditidae family with fewer proportions of other entomopathogenic opportunists (e.g., *Oscheius* spp., Bisel *et al*., 2016). The virulent strains after three subsequent re-inoculations consisted of *Heterorhabditis* spp. alone (Rhabditida: Heterorhabditidae).

*Pathogenicity of EPNs on CRB larvae*

Among the EPN strains tested, Moku-3, Lyon-4, and Keaau-3 inflicted larval mortality after 2 days, whereas Hoo-2, Moku-3, Lyon-2, and Keaau-3 inflicted mortality after a week of exposure. There was slight larval mortality [2.5 ± 2.5% (after 2 days) and 12.5 ± 6.3% (after a week)] in the control treatment, but the reason for this mortality was not clear (Fig. 2). The effects of EPN strains on larval mortality were not significant at both times [$F_{9,15}$ = 0.93 - 2.38, $P$ > 0.05]. However, the percentage mortality was greatest with strains Keaau-3 [15.0 ± 5.0] after 2 days and Lyon-2 [34.3 ± 5.7] after a week of exposure. Keaau-3 was the most consistent strain in causing mortality after 2 days [15.0 ± 5.0%] as well as a week of exposure [25.0 ± 5.0%].

## DISCUSSION

This study showed that *Heterorhabditis* spp. are widely distributed in various landscape systems on the island of Oahu. A survey conducted on all Hawaiian Islands by Hara *et al*. (1991) found that the composition of EPNs at the site primarily depended upon soil type and distance away from the seashore. Heterorhabditids were abundant on beaches within 100 m of seashore (sandy soil), whereas Steinernema were found in the inland areas (silty clay to silty loam) (Hara *et al*., 1991).

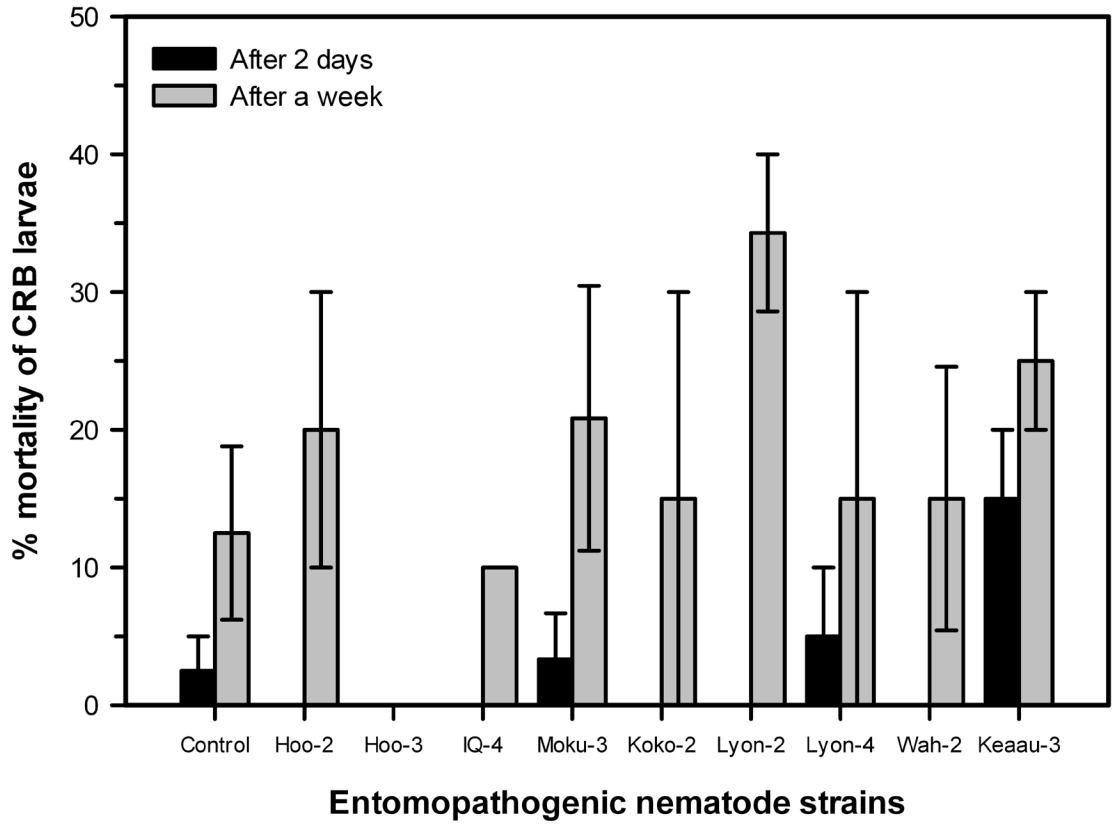


Figure 2. Percentage mortality of first instar coconut rhinoceros beetle (CRB) larvae when exposed to various strains of *Heterorhabditis* spp. for two days and a week.

A survey on occurrence and distribution of Heterorhabditid populations on coastal areas in the Hawaiian Islands showed that *Heterorhabditis indica* and *Heterorhabditis* spp. were widely distributed on all islands except Molokai (Myers *et al*., 2015). In the current study, the survey sites were randomly distributed around the island with similar soil texture (sandy-loam to clay-loam). Our survey confirmed *Heterorhabditis* spp. can be easily isolated from the soils of various landscape sites, irrespective of their location either in coastal areas or inland. This supports the prediction made by Hara *et al*. (1991) that *Heterorhabditis* may have been brought to Hawaii through the ballasts of ships and established in the new habitat (coastal areas). Over time, the distribution of EPNs could have extended inland potentially from the movement of insect hosts and humans moving sand inadvertently or deliberately from the beach parks where *Heterorhabditis* were previously recovered.

Studies have shown that *Heterorhabditis* spp. can cause significant mortality in white grubs of Scarabaeidae family both in lab and field conditions. For example, the efficacy of *H. bacteriophora* was comparable to that of applications of diazinon against Chafer beetle, *Cyclocephala hirta* and Japanese beetle, *Popillia japonica* larvae under field conditions (Koppenhofer *et al*., 2000). Grewal *et al*. (2004) found two virulent EPN stains, *H. zealandica* X1 and *H. bacteriophora* GPS11 through laboratory bioassays that controlled two white grub species, *P. japonica* and *C. borealis* up to 98% in field trials. Unlike these studies, our study did not show significant effects of *Heterorhabditis* spp. strains on CRB larvae mortality. However, some strains (Moku-3, Keaau-3, and Lyon-4) caused mortality of first instar larvae after 2 days as well as after a week of exposure. Thus, our findings indicated that the locally available strains of *Heterorhabditis* spp. could potentially be a useful biological control agent, in an IPM program, for controlling first instar CRB. However, further research is needed to test virulent commercial strains of Heterorhabditid and Steinernematid after import permits of these biological control agents are obtained from HDOA. The results of such studies will uncover if certain EPN strains could be incorporated as a part of CRB IPM program.

Literature suggests that little is known about the host range and pathogenicity of *Heterorhabditis* spp. in Hawaii (Hara *et al*., 1991; Myers *et al*., 2015). As indicated by these authors, we also did not note many hosts, except cockroaches, earthworms, and collembolans during the survey. It would be interesting to explore how EPNs persist in these ecosystems without the availability of host insects. However, increasing incidence of soil insect pests (e.g., oriental flower beetle, sod webworm, stag beetle, CRB) may enhance the population distribution of EPNs in Hawaii. Then, EPNs, in turn, may suppress susceptible pest hosts and provide biological control services in soil ecosystems in Hawaii.

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