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Full Length Research Paper

Isolation of entomopathogenic nematodes and control of *Phyllophaga vetula* Horn in Oaxaca, Mexico

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This study aimed to isolate native entomopathogenic nematodes (EPN) in the Central Valleys of Oaxaca and to determine their potential for control of white grub (*Phyllophaga vetula* Horn). Fifty-five (55) soil samples were collected in 13 communities in the period August to October 2008 and 29.1% of these were found positive for EPN. Five isolates were selected for their apparent pathogenicity to third instar *Galleria mellonella*; two of the genus *Steinernema* and three *Heterorhabditis*. After evaluating these five isolates for control of white grubs, the Heterorhabditidae EPN presented the lowest values for both lethal dosages and lethal times. Using polymerase chain reaction (PCR) procedures, the species *Heterorhabditis mexicana*, *Steinernema carpocapsae* and *Steinernema feltiae* were identified. We concluded that *H. mexicana* was the most effective isolate for control of *P. vetula* larvae.

Key words: Entomopathogenic nematodes, *Steinernema, Heterorhabditis*, bioassays, lethal dosages.

INTRODUCTION

In the Central Valleys of Oaxaca Region, some 110,000 ha are planted with maize and Coleoptera larvae (white grubs) can cause severe plant damage. There are no available data to evaluate the extent of the problem, but severely infested plots decrease crop yields more than 50% (400 to 700 kg ha^{-1}), mostly because of reduction in plant stand (Ruiz et al., 2003). Bravo (2003) found grain yield increases of 300 to 375 kg ha^{-1} when an insecticide was applied to plots infested with white grubs. Villalobos (1992) estimated maize yield reductions in the order of 400 to 1300 kg ha^{-1} in Central Mexico. In Oaxaca, most of the grain produced is for self consumption and since it is a low-input agriculture, the farmers require a low-cost, yet effective method to control white grubs.

Entomopathogenic nematodes (EPN) are obligate parasites in nature, which gives them the possibility of being used as bio-control agents and therefore represent a good alternative to chemical insecticides (Kaya and Gaugler, 1993); the third stage (infective juvenile) emerges from the host's body and can remain for months in the soil until it finds a new host (Kaya and Koppenhofer, 1996). Their hosts are larvae known as "white grubs" (Coleoptera: Scarabeidae), which are important pests of various crops, including maize and root crops (Koppenhofer, 2007).

It is recommended that native species are isolated, thereby increasing the chances of successful control; however, their effectiveness also depends on environmental factors such as soil moisture (Koppenhofer et al., 1995), temperature (Grewal et al., 1994), soil texture, and bulk density (Gruner et al., 2007).

The use of biological control agents cannot be generalized, but experimentation is required to determine which kind of NE is most suitable for controlling a certain species of white grubs (Koppenhofer, 2007). Therefore, when a new NE is isolated from field samples, laboratory assessments of ineffectiveness and control efficacy have to be done before applying it in the field (Wang and Grewal, 2002).

Also, it is important to know the host range and lethal dosages of the EPN, as well as their interactions with pest populations. This knowledge is necessary to use them effectively as an alternative to synthetic pesticides (Jackson, 2003).

The EPN of the families Heterorhabditidae and

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Steinernematidae have great potential as biological control agents of many insect pests (Griffin et al., 2005). These nematodes possess many desirable attributes, including wide host range, safety for non-target organisms and the environment, exemption from registration in many countries, easy mass production and application, ability to find the pest, and potential to persist in the environment, among others (Kaya and Gaugler, 1993).

The present study was aimed to isolate EPN from soil samples collected in 13 locations of the Central Valleys of Oaxaca Region, Mexico. The isolates were evaluated for their potential to control white grubs (*Phyllophaga vetula* Horn), and Probit Analysis (SAS, 2002) was used to determine the lethal dosages and times to control this soil dwelling pest.

MATERIALS AND METHODS

Soil sampling

To detect and isolate local EPN species, soil sampling was carried out in the Central Valleys of Oaxaca Region, which has a mean annual temperature of 22.0°C and 650 mm rainfall, and is located at altitudes between 1500 to 1600 m. The sampling period was during the months of August to October, when the maize crop was in the reproductive stages and was actively growing because of adequate soil moisture, which increased the chances of finding EPN.

The presence of white grubs in the previous growing seasons was one of the criteria for choosing the sampling sites, as well as visible crop symptoms indicating the presence of the pest; theoretically, where there is a specific pest, there is also a natural enemy, which makes it an intentional sampling process (Azoríny and Sánchez, 1986).

Using a straight shovel to reach a depth between 10 and 30 cm, to discard the drier top layer, one sample (~1 kg of soil) per site was taken at about 10 cm from the base of the corn stalk; after homogenization in the shade to avoid temperature increases and solar radiation, which could affect the nematodes present in the sample, they were placed in a plastic bag to prevent moisture loss, and stored in a cool chest. After taking each sample, the blade was washed with water to prevent possible mixing of nematodes between sampling sites. A total of 55 sites were sampled.

Once in the laboratory, each 1 kg soil sample was placed in a plastic tray (20 × 15 × 7 cm) and ten late instar *Galleria mellonella* larvae were applied to each one, afterwards the tray was covered with aluminum foil to prevent moisture losses, but small holes were made to allow for gas exchange.

Every two days, over a period of two weeks, the trays were inspected to determine whether there were dead larvae; these were placed in White Traps (White, 1927) and when there was emergence of infective juveniles (IJ's), they were harvested and stored in properly labeled culture flasks at an average temperature of 10°C.

Experiment 1: Verification of entomopatogenicity

An experiment to verify the entomopathogenicity of the isolates was carried out. Petri dishes were lined with filter paper and the 27 isolates were separately applied in an aqueous suspension at 300 IJ's per dish. Afterwards, 10 *G. mellonella* larvae were placed in each dish, which were placed inside plastic bags to prevent desiccation of the nematodes. After five days, the percentage of larval mortality was determined and each larva was placed in a White trap to verify their ability to produce IJ's in adequate numbers. This experiment was laid out in a completely randomized design and replicated four times.

Experiment 2: Lethal dosages and times for selected isolates

Using 1/3 of the most effective isolates detected in the bioassay with *G. mellonella*, five dosages: 50, 100, 200, 500 and 1000 IJ's per larva were evaluated. Also, a blank treatment without application of IJ's was included. There were 10 cups per treatment and the experimental design was a randomized one with four replicates. The plastic cups (100 ml) were previously filled with dry, sterilized soil to eliminate the influence of other pathogens. The soil used was loamy sand and was maintained at 80 ± 5% field capacity (FC). The water content at FC of this soil was 17.3%. Thus, to maintain the soil at 80% FC, 13.8 ml of distilled water was added initially. Every three days the cups were weighed and more water was added as needed to reach 80% FC again.

Each day after water was added one *P. vetula* larva was placed in the soil surface of each cup; after a waiting period of 30 min, non-active larvae were discarded, and the five different dosages of nematodes were applied in an aqueous suspension (200 µL). In order to avoid a rapid loss of moisture, the cups were covered with aluminum foil and a small hole was made with a toothpick.

Every other day, for 16 days, the cups were opened to determine the number of dead larvae. To determine whether the larva had actually been killed by a nematode infection, they were placed in White traps. Using the data from the tenth day after the start of the experiment, the lethal doses were determined, whereas all data collected during the duration of the experiment were used to determine the lethal times; both indicators were established for the control of 50 and 95% of the grub population.

The experiment was established under a completely randomized design and four replicates. Before data analysis, the mortality percentages were transformed with the arcsine square root procedure. The analysis of variance and Tukey's test ($\alpha = 0.05$) for the separation of means was carried out with the Statistical Analysis System package (SAS, 2002).

Determination of the species of nematodes

The following procedure, the selected nematodes were identified by polymerase chain reaction (PCR) techniques (Ellis et al., 1986; Zhou et al., 1998) in the Nematology Laboratory of The University of California, Davis.

DNA extractions

Individual adult nematodes were dissected from infected *G. mellonella* cadavers, cut in half and placed in a mix of 20 µl 'Chelex 100' resin (5%, Bio-Rad, Hercules, CA), and 1 µl proteinase K solution (20 mg/ml; Sigma, St. Louis, Missouri). The mixture was incubated at 56°C for 1 h, 100°C for 8 min, and then cooled to 40°C for 30 s. After vortexing for 30 s, samples were stored at -17°C. In cases where adult nematodes could not be found, individual infective juveniles were used.

PCR conditions

The ITS-1 and ITS-2 regions of ribosomal DNA were amplified using 18S and 28S primers (no. 93, 5' TTGAACCGGGTAAAAGTCG and no. 94, 5'TTAGTTTCTTTTCCTCCGCT) designed by

Table 1. Results of soil samplings for entomopathogenic nematodes (EPN) conducted in the Central Valleys of Oaxaca, from August to October, 2008.

Locality	No. of samples	No. of positive samples and non-EPN	Positive samples for EPN (%)
Cuilapam de Guerrero	9	4	44.4
Santiaguito, Etla	2	1 (1)[¥]	50.0
Guadalupe, Etla	6	2 (1)	33.3
Zaachila	4	1 (1)	25.0
Santa Cruz Xoxo	3	2 (1)	66.7
San Dionisio, Ocotlán	4	1	25.0
Lachigoló, Tlacolula	1	1	100.0
Reyes Mantecón	8	0	0.0
Col. Emiliano Zapata	2	2	100.0
Sta. Inés Ayoquezco	3	2	66.7
Sn. Pedro Mártir	4	4 (2)	100.0
Los Ocotes, Ejutla	4	4 (2)	100.0
San pablo Etla	5	3 (3)	60.0
Total	55	27 (11)	49.1

[¥]Number of non-entomopathogenic samples.

Table 2. Isolates with higher rates of mortality on *Galleria mellonella*.

Sample number	Locality	Probable genera	Mortality (%)	Code
1	Cuilapam	*Steinernema*	97.5	CS1
2	Cuilapam	*Steinernema*	100	CS2
5	Cuilapam	*Heterorhabditis*	95	CH5
8	Cuilapam	*Heterorhabditis*	100	CH8
24	Lachigoló Tlacolula	*Heterorhabditis*	100	LH24

Nadler et al. (2000). PCR amplification was performed in a reaction volume of 25 µl, containing 2.5 µl of 10x PCR-buffer, 14.6 µl of H_2O, 3 µl of $MgCl_2$ (25 mM), 0.5 µl of dNTPs (10 mM), 0.4 µl Taq polymerase (5 units/µl, Gene choice T-18), 1.25 µl of primer forward (10 µM), 1.25 µl of primer reverse (10 µM), and 1.5 µl of DNA. All PCR reactions were conducted in an Applied Biosystems 2720 thermocycler with a profile of: 1 cycle at 94°C for 2 min followed by 35 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 50 s. The last step was a post-amplification extension at 72°C for 4 min.

Sequencing

Amplified products were purified using enzymatic treatment with exonuclease I and shrimp alkaline phosphatase (PCR product pre-sequencing kit, USB Corporation). Primers used for sequencing were the same as for PCR. Contiga were assembled using the program Codon Code Aligner (Version 3.6.1) and a basic local alignment search tool (BLAST) search performed on the final consensus sequence. Only matches above 99% identity were considered positive (Stock et al., 2001).

RESULTS

Soil sampling

Table 1 shows the results of the soil samplings conducted in 13 communities of the Central Valleys of Oaxaca. From a total of 55 samples taken, 27 were positive for nematode infection, that is, 49.1% of the samples taken were infested by nematodes, but not necessarily entomopathogenic. According to the color of the dead larvae, at this point, 81% were infected by EPN of the genus *Steinernema* and 19% by *Heterorhabditis*.

Kaya and Stock (1997) mentioned that the *G. mellonella* larvae infected with EPN of the genus *Heterorhabditidae* may have reddish-orange coloring, while those infected with *Steinernema* spp. show gray-brown coloring. *G. mellonella* cadavers infected by different EPN isolates, showed distinctive colors, those infected by *H. bacteriophora* and *H. indica* were brick red, while those infected by *Steinernema feltiae* were brown in color (Nyamboli, 2008) (Table 2).

Experiment 1: Selection of entomopathogenic isolates

After isolation, the nematodes were bioassayed on *G. mellonella* larvae to detect the entomopathogenic ones. Eleven samples did not produce IJ's in adequate numbers or did not produce them at all, mostly because

Table 3. Analysis of variance of transformed *Pyllophaga vetula* larvae mortality data.

Source	Degrees of freedom	Sum of squares	Mean squares	F value	P>F
Model	24	28.35	1.18	43.11	<0.0001
Error	75	2.05	0.02		
Total	99	30.40			
Treatments	24	28.35	1.18	43.11	<0.0001
Isolates	4	0.13	0.03	14.35	0.0258
Dosages	4	32.32	8.13	485.12	<0.0001

R^2 = 0.91; C. V. = 14.02%

of fungal infection. Thus, the percentage of samples found positive for EPN was 29.1%.

About 1/3 of the isolates conformed a group where the control percentage was 95 to 100%. Four of the selected isolates were collected in the community of Cuilapam, Zaachila; these caused larval fatalities ranging from 95 to 100%. The other selected one was collected in the town of Lachigoloo, Tlacolula, which produced 100% mortality. The range of mortality observed for the remaining 10 isolates was 35 to 80%.

Experiment 2: Lethal dosages and times for selected isolates

The analysis of variance of transformed mortality data of *P. vetula* larvae caused by five of the most effective isolates are shown in Table 3. The variance analysis showed that the general statistical model was adequate ($P < 0.05$); the R^2 value was 0.91, indicating that the model explained 91% of variability of the experiment.

Treatments and dosages showed highly significant effects ($P < 0.01$), but isolate's effects were only significantly different ($P < 0.05$). Thus, there were statistical differences in at least one level of these factors. The comparison of means between treatments, and nematodes and dose factors are presented in Tables 4, 5 and 6, respectively. Table 4 shows that the main factor determining the statistical groups was the number of IJ's applied, as all the five isolates evaluated with 1000 nematodes per larva showed a high mortality. There was a clear statistical difference between the three higher dosages, but the two lower ones had no statistical differences between them (Table 5).

Table 6 shows that the nematodes from Heterorhabditidae, as compared with *Steinernema*tidae, are grouped in a different statistical group with a more effective grub control. Table 7 shows the lethal dosages and times for each of the five isolates applied for control of *P. vetula*. Both 50 and 95% dosages form two groups, one for the Heterorhabditidae, which require lower dosages to control white grub larvae, and another for the *Steinernema*tidae with higher dosages for control. The same groups are also valid for lethal times, where the *Steinernema*tidae showed the longer periods to control the larvae.

Determination of the species of nematodes

The isolates CH8, LH24 and CH5 were identified as *Heterorhabditis mexicana* Nguyen, Shapiro-Ilan, Stuart, McCoy, James and Adams; while isolate CS2 was identified as *Steinernema carpocapsae* Filipjev. The CS1 isolate was identified as *S. feltiae* Filipjev.

DISCUSSION

The percentage of positive samples for nematodes obtained in this study was 29.1%, which might seem high when compared with other studies, such as Stock et al. (1999), who conducted a sampling of different habitats in the state of California and found 26.3% of positive samples. In Oaxaca, Ruiz-Vega et al. (2003) found a maximum of 8.9% of positive samples in the region. The difference with the last study mentioned above may be because the sampling was random, including natural vegetation areas, while in the current study sampling was directed to maize fields where white grub infestation had occurred and thus the presence of EPN was more likely. This idea is supported by the study of Ruiz-Vega et al. (2003), who found that the highest percentages of nematode soil samples were found where the grubs were present.

In terms of percentage of positive samples by genera, this study found 68.7% of EPN in the genus *Steinernema* and 31.3% in the *Heterorhabditis*, which concurs with Stock et al. (1999), who reported 80% in *Steinernema* and 20% in *Heterorhabditis*; on the other hand, Ruiz-Vega et al. (2003) found 67% in *Steinernema* and 33% in *Heterorhabditis*.

The isolated nematodes were subject to an evaluation of relative pathogenicity. Those who were attacked by fungus or produced IJ's in small numbers were considered not entomopathogenic. It is known that when

Table 4. Comparison of percent mortality means of *Phyllophaga vetula* between treatments.

Treatment	Nematode species	Dosage (IJ's/larva)	Mortality (%)
25	*H.* mexicana	1000	100[a¥]
15	*H.* mexicana	1000	100[a]
10	*S. carpocapsae*	1000	97.5[ab]
20	*H.* mexicana	1000	97.5[ab]
5	*S. feltiae*	1000	95[b]
14	*H.* mexicana	500	90[c]
24	*H.* mexicana	500	90[c]
19	*H.* mexicana	500	87.5[cd]
18	*H.* mexicana	200	87.5[cd]
4	*S. feltiae*	500	47.5[e]
9	*S. carpocapsae*	500	47.5[e]
13	*H.* mexicana	200	37.5[f]
23	*H.* mexicana	200	37.5[f]
8	*S. carpocapsae*	200	32.5[g]
3	*S. carpocapsae*	100	30.0[g]
12	*H.* mexicana	100	22.5[h]
7	*S. feltiae*	200	17.5[i]
22	*H.* mexicana	100	15.0[ij]
21	*H.* mexicana	50	15.0[ij]
17	*H.* mexicana	100	10[k]
1	*S. feltiae*	50	7.5[kl]
16	*H.* mexicana	50	5.0[l]
2	*S. feltiae*	100	5.0[l]
11	*H.* mexicana	50	5.0[l]
6	*S. carpocapsae*	50	5.0[l]

[¥]Values with the same letter are not significantly different (Tukey P = 0.05).

Table 5. Comparison of mean mortality percentages of *Phyllophaga vetula* for different dosages of the EPN isolated.

Dosage (IJ's/larva)	Mortality (%)
1000	98.0[a¥]
500	72.5[b]
200	42.5[c]
100	16.5[d]
50	7.5[d]

[¥]Values with the same letter are not significantly different (Tukey P = 0.05).

Table 6. Comparison of mean mortality percentage of *Phyllophaga vetula* for the different nematode species isolated.

Isolate species	Mortality (%)
H. mexicana	57.5[a¥]
H. mexicana	51.5[a]
H. mexicana	51.0[a]
S. carpocapsae	42.5[b]
S. feltiae	34.5[c]

[¥]Values with the same letter are not significantly different (Tukey P = 0.05).

an insect is infected with the nematode's symbiotic bacteria, it is protected against the attack of other organisms. Rosa et al. (2000) consider that when larvae are infected by EPN, these will not rot, nor have foul odors. Also, each host will produce IJ's in large numbers. However, temperature and inoculums level have been found to affect the amount of IJ's produced.

In an evaluation with *H. heliothidis* and *S. glaseri*, temperature and inoculum level significantly affected time to first emergence, duration of emergence, and yield of juveniles from *G. mellonella* larvae (Zervos et al., 1991). Sepúlveda-Cano et al (2008) found similar results for inoculum level as they had a larger number of IJ's for 1000 than for 100 JI/25 µl; the amounts of IJ's produced per *Cosmopolites sordidus* larva was 99,386 for *H. bacteriophora* and 106,532 for *S. carpocapsae*. These numbers agree with Realpe-Aranda et al. (2007), who obtained an accumulated production of 86,250 IJ's/larva for *Steinernema colombiense* and 78,750IJ/larva for *H. bacteriophora.*

Table 7. Dosages (D_{50} and D_{95}) and lethal times (LT_{50} and LT_{95}) for five nematode species isolated for control of *Phyllophaga vetula*.

Isolate species	LD_{50}	LD_{95}	LT_{50}	LT_{95}
H. mexicana	326	726	3.1	7.1
H. mexicana	339	602	2.7	6.2
H. mexicana	348	613	2.8	6.3
S. carpocapsae	382	920	4.0	9.8
S. feltiae	405	998	4.1	10.3

According to the general statistical model, the more important factor to generate significance was the dose of nematodes applied, followed by isolates. In relation to application rates, the best dose was 1000 nematodes per larva, which coincides with Athanassiou et al. (2010), who reported that using *S. feltiae* in laboratory bioassays for the control of *Tribolium confusum* larvae Jacquelin du Val, the dose that caused the highest mortality (up to 56%) was 1000 nematodes per larva. Trdan, et al. (2009) found that for the control of larvae of Colorado potato beetle (*Leptinotarsa decemlineata),* the best dosages were 1000 and 2000 nematodes per larva.

The Heterorhaditidae were grouped in a different statistical group than the *Steinernema*tidae, the first being more effective for white grub control. The Heterorhabditidae had the lowest lethal doses; LD_{50} dosages varied from 326 to 348, while LD_{95} dosages ranged from 602 to 726 IJ's per larva. However, for the *Steinernema*tidae LD_{50} dosages varied from 382 to 405 and LD_{95} dosages varied from 920 to 998 IJ's per larva. Rodríguez et al. (2009) found an LD_{50} of 475 nematodes per larva for a native isolate of *Heterorhabditis* sp. applied to second and third stage larvae of *Phyllophaga elenans*, which is similar to the one found in the present study.

Also, the Heterorhabditidae presented the lowest LT_{50} values, ranging from 2.7 to 3.1 days, and the LT_{95} varied from 6.2 to 7.1 days; while for the *Steinernema*tidae the LT_{50} ranged from 4.0 to 4.1 and the LT_{95} had values of 9.8 to 10.3 days. Rodríguez et al. (2009) found LT_{50} values of 7.5 days for 625 IJ's of *Heterorhabditis* sp. applied to larvae of *P. elenans*. Ruiz-Vega et al. (2003) found an LT_{50} of three to seven days for *S. carpocapsae* and *S. feltiae* applied to *P. vetula* larvae.

Conclusions

This is the first report of *H. Mexicana* in the area, while *S. feltiae* and *S. carpocapsae* had been reported previously (Ruiz-Vega et al., 2003; Aquino et al., 2006). *S. feltiae* was located in a *Quercus spp.* forest, while *S. carpocapsae* was found in a plantation of *Agave angustifolia Haw.*

H. mexicana can be considered to show the highest percentage of control, but at 57.5%, this is still considered a moderate effectiveness. According to Georgis et al. (2006), a good control potential is above 70% effectiveness. Shapiro-Ilan et al. (2005) concluded that *H. mexicana*, relative to other entomopathogenic nematodes, generally possesses moderate abilities in bio-control traits. *H. mexicana* showed a medium potential for control of *P. vetula*, while *S. carpocapsae* and *S. feltiae* showed a low to very low potential.

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