Modelling movements of root-knot nematodes *Meloidogyne* spp. juveniles when encumbered with spores of *Pasteuria penetrans*

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Abstract. A system for monitoring movement of the free-living second stage juveniles of root-knot nematodes (*Meloidogyne* spp.) using digital image analysis is described. The method is based on the analysis of video sequences of movement of individual random nematodes encumbered with or without *Pasteuria penetrans* endospores. Software packages were used to grab video images, to process images and to monitor the movement of selected body part positions over time. Methods include the study of nematode locomotion based on (a) the geometric center (b) the centroid body point, (c) tracking of two or four selected body points and (c) tracking a rectangular shape area produced by the nematode's body. Data showed that (a) the normal sinusoidal movement of nematodes is changed when individuals are encumbered with spores of *P. penetrans* and (b) in all cases a significant greater motility was observed by nematodes without *P. penetrans* spores attached.

Keywords: Nematode movement**,** digital image analysis, motion analysis.

1 Introduction

Pasteuria penetrans (Thorne, 1940) is a mycelial, endospore forming bacterial parasite of plant parasitic nematodes (Mankau, 1975; Imbriani and Mankau, 1977) showing promising results in a biocontrol strategy of root-knot nematodes (*Meloidogyne* spp.) (Stirling, 1991). The endospores attach to the outside nematode body wall (cuticle) of the infective stage, the second-stage juveniles (J2) of *Meliodogyne* populations (Mankau, 1980). After the J2 penetrates a plant root and begins to feed, the bacterium penetrates the nematode body wall and begins to grow and develop in the developing nematode (Mankau and Imbriani, 1975; Imbriani and Mankau, 1977; Sayre and Wergin, 1977). Eventually, the female nematode body becomes completely filled with spores (Sayre and Wergin, 1977; Stirling, 1991). Each infected female may contain up to 2.5 million spores (Darban *et al.*, 2004), which are eventually released into the soil.

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The potential of *P. penetrans* to control of root-knot has been widely studied (Gowen *et al*., 2008) including distribution, host range, and specificity. Successful parasitism depends on the attachment of 5-10 spores per juvenile, which is sufficient to initiate infection without reducing the ability of the nematode to invade roots (Davies *et al.*, 1988; Rao *et al.,* 1997). There may be little or no root invasion if there are greater than 15 spores attached, inferring that spore attachment will affect the ability of a J2 to locate and/or invade a root (Davies *et al.,* 1988). Few attempts have been made to quantify the effect of *P. penetrans* spore attachment on the movement of infective root-knot nematode juveniles.

Nematodes move by undulations or wave-like motions through dorsal/ventral contractions of the body (Buchsbaum *et al.,* 1987; Storer *et al.,* 1979) similar to an undulatory swimming motion of eels (Tytell, 2004) and as larval chironomids (Brackenbury, 2003). As one segment of the body contracts, it "pulls" the remainder of the body forward along the body in a head to tail direction (Brackenbury, 2000). There are no previous experimental data to model root-knot nematode movement encumbered with or without *P. penetrans* spores. In this paper a study using digital image analysis is made of the movement of the root-knot nematode (*Meloidogyne* spp.) and how this is affected when sporesof the bacterium *Pasteuria penetrans* (a naturally occurring nematode parasite) are attached to the cuticle.

2 Materials and methods

2.1 Nematode cultures

Nematode cultures (*M. javanica*) were maintained on tomato plants in the glasshouse and fresh second stage juveniles (J2) were collected from infected tomato roots using the methods described by Hussey and Barker (1973).

2.2 Preparation of Pasteuria penetrans spores

A commercial product of *Pasteuria penetrans* (Pp) (Nematech Co. Ltd., Japan) was used in this study. Fresh J2 were encumbered with Pp spores as described by Darban *et al.* (2004). Nematodes with 5-10 spores attached were considered as the Low Pp and J2 with 15-25 spores were the High Pp treatment.

2.3 Acquisition of the video images

Nematode locomotion was tracked with an inverted microscope (MICROTEC 200) mounted with a digital camera (Aptiva 3.2 Megapixel). In all cases a nematode's movement was observed in water in a 9 cm Petri dish. Nematode movement was recorded in 30 second video sequences. The microscope magnification was x 100 for Figure 1, x 200 for Figures 5, 6 and 7 and the highest magnification for Figure 3. All video sequences showing nematode locomotion were observed on Movie Maker 2 (Microsoft software) before any further analysis.

2.4 Image extraction

Before analysis, images (frames) grabbed from selected 30 sec videos were obtained. We used a video decompiler (SC Video decompiler software program) to extract

frames. A total of 390 frames saved in jpg format were obtained from a 30 sec video. Original images were 320 x 240 pixels or 3.3 x 2.5 inches.

2.5 Measurement of nematode movement

Measurement of movement (in inches) was performed using image analyzer software Scion Image for Windows (Scion Corporation, www.scioncorp.com).

2.6 Image processing and analysis

All frames were saved in a tiff format before importing to the Scion Image software package. For image analysis the 39 frames were aligned in ranks of 10 as the 1st, 11th, 21st and the 31st frame. When an image file (*.tiff) was imported to the Scion Image software program, measurements were performed with the Manual Area Measurement selecting the Measure command of the program. In order to understand the aspects of nematode body posture, measurements were taken of the body movement. For each frame, five measurements were taken; (a) tracking of the nematode geometric center; (b) tracking the nematode centroid body part; (c) tracking of two or four selected body points and (d) tracking a rectangular shape area produced by the nematode's body. In detail, the measurements of the five above measurements are:

2.6.1 Nematode locomotion

Using the rectangular selection tool of the Scion Image Program and moving the mouse on the nematode image, we fitted all faraway nematode body segments in a rectangle shape in order to estimate the X-Y geometric center (using the measurement options Area, X-Y Center of the Scion Image program). With the geometric center (X-Y center of the rectangle area) we were able to track the geometric point of the nematode body, or very close area matches in that point. Further, we extracted metrics to an Excel data spread-sheet and presented all data to GenStat 7th Edition to create a scatter plot. The trends of two individual J2/treatment movement directions were randomly selected and shown in a scatter plot.

2.6.2 Tracking the nematode centroid point and two or four nematode body points

The centroid points, of four random J2 per treatment were tracked using the Elliptical Collection Tool of the Scion Image Program follow the procedure described above.

Using the Cross Hair Tool, and moving the mouse on the nematode image we marked four equal-distance nematode body points and their X-Y coordinates were recorded for each frame and analysed in the Program Results window. The points were: 1 the head; 2 the esophagus; 3 the centre of the gut: 4, the tail. All data were extracted as X and Y values on the program Result window and exported to an Excel data spread-sheet.

Based on those data we displayed the nematode per treatment movement, with the centroid body point and the relationship of four nematode body parts or the relationship of the two intermediate (Ym1 and Ym2) nematode body points movement in all Pp treatments.

Moreover, the equation of nematodes movement based to the centroid point were obtained by fitting the data sets (X, Y values) to CurveExpert 1.3, a curve fitting system for Windows by using the Program CurveFinder command.

Further, the equation of nematodes movement based to four or to two intermediate Ym1 and Ym2 nematode body parts were obtained by fitting the data sets values to GenStat by using the Standard Curves command of the Nonlinear Regression Analysis.

2.6.3 Tracking the nematode rectangular shape area
Using the Rectangular Selection Tool of the Scion Image Program and moving the mouse on the nematode image, we fitted all faraway nematode body segments in a rectangular shape in order to estimate the rectangular shape area, using the measurement options Area of the Scion Image Program. Further, we extracted measurements to Excel and represented the different rectangular shaped areas produced by nematodes encumbered or unencumbered with Pp endospores.

2.6.4 An estimation of J2 motility
The locomotion of nematodes treated with low and high or without *P. penetrans* spores were estimates based on the nematode wavelength (λ) and the distance moved over time. The body lengths of each nematode were measured using the Straight Line Selections Tool of the Scion Image Program and moving the mouse on the nematode image marked the nematode head and the tail each time. The same procedures were used to measure the distance covered by the nematode head over time t1 and t2 (frames 1 and 2) (up to 15 frames) moving the mouse from X1,Y1 (frame 1) to X2,Y2 position on frame 2. Data were exported to an Excel data spread-sheet as described above. The absolute body length of a J2 in this research is equal to a 105 pixels as presented in Figures 6 and 7 with a dotted line. Measurements were based on 10 nematodes per treatment and with 15 frames per individual nematode. The time between two frames in sequence was 11.5 sec.

2.7 Statistical analysis
All regression analyses were performed to GensStat 7th Edition statistical program. Scatter line plots (Figures 1-3) were performed to MinTab 13th Edition statistical program and all other graphs were made to GraphPad Prism5 statistical program.

3 Results

3.1 Nematode locomotion
Over the same time period, J2 without *P. penetrans* spores attached can move further than those J2 encumbered with low numbers of spores (Figure 1). That means that the body of a J2 without Pp spores moves in a direction explained by a linear regression r^2 96-98%. Attachment with *P. penetrans* spores probably disrupts this natural behaviour.

3.2 Tracking the nematode centroid point and two or four nematode body points
When data sets based on the nematode centroid body point were fitted to CurveExpert, the best equation to explain the J2 body movement without *P.*

penetrans spores(Figures 2.1a-2.4a) is the sinusoidal fit, y=a+bcos(cx+d) with correlation coefficient r1=0.9368, r2=0.9396, r3=0.9345 and r4=0.9090 (R^2 parameters based to four random nematodes). When nematodes were encumbered with *P. penetrans* sporesthere was no sinusoidal movement (Figures 2.1b-2.4b and Figures 2.1c-2.4c).

Our simple tracking system for extracting data from the four or the two intermediate nematode body part points has shown that nematodes without *P. penetrans* spores attached have a sinusoidal movement producing a double Fourier curve (equation 1). This is represented in Figure 3A where the Ym1 line presents the esophagus and the Ym2 line the gut ($R^2_{Ym1+Ym2}$ = 86.0, P<0.001) and in Figure 3B where the tracking points are the head, the esophagus, the centre of the gut and the tail ($R^2_{head+esophagus+gut+tail}$ = 78.4, P<0.001).

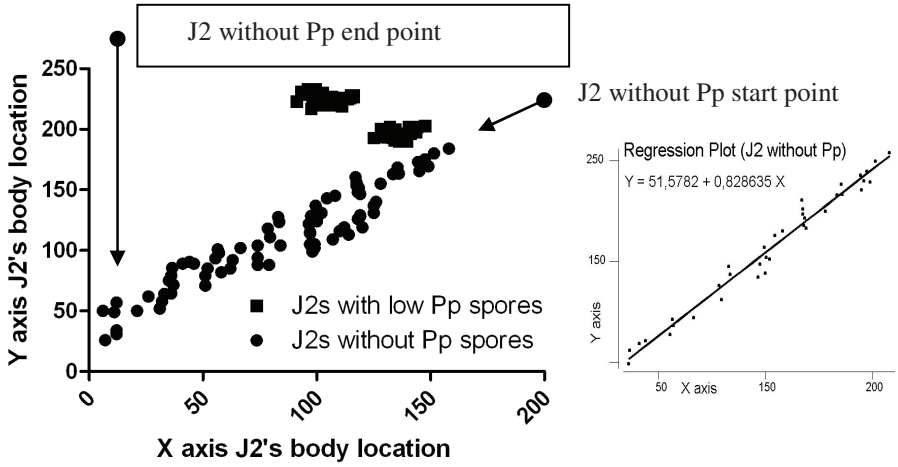

Fig. 1. Nematode travel position without *P. penetrans* spores (circle solid dots) and with low *P. penetrans* spores (square solid dots, top). The arrows indicate the direction of movement (X, Y axis units in pixels). The nematodes without *P. penetrans* spores move in a line explained by a Linear regression fit y=a+bx with a correlation coefficient (R^2) equals to 0.9868 and 0.9679 for the two random individual nematodes. Axis scales x, y are measured in pixels (200_250 pixels), value for a straight J2 body length is 10 pixels.

Based on the movement of the four body points, the best curve to explain our data is also the double Fourier curve. This is a compound of two sine waves as presented in Figure 4, one having half the cyclic period of the other.

$$Y = a + b*\sin(2*pi*(X-e)/w) + c*\sin(4*pi*(x-f)/w) \qquad \text{equation 1}$$

A J2 free of Pp spores fits a rectangle shape with a forward movement* (figures 1-4a in a column)

A J2 encumbered with low Pp spores fits in a smaller box with a slow movement (figures 1-4b in a column)

A J2 encumbered with high Pp spores, fits in box with no forward movement (figures 1-4c in a column)

Box a

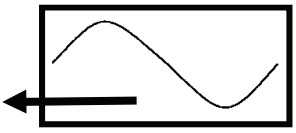

Figure 2.1a, t=0sec

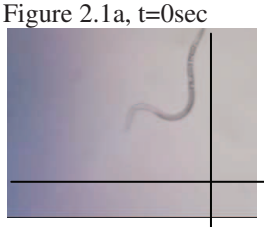

Figure 2.2a, t=10sec

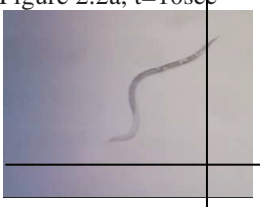

Figure 2.3a, t=20sec

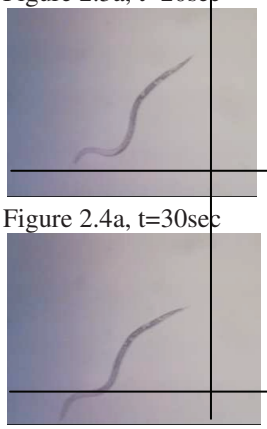

Figure 2.4a, t=30sec

Box b

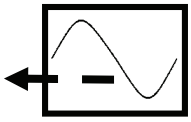

Figure 2.1b, t=0sec

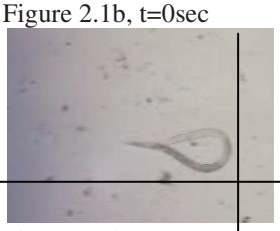

Figure 2.2b, t=10sec

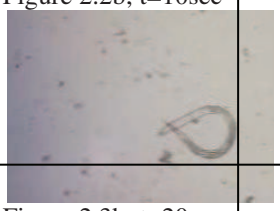

Figure 2.3b, t=20sec

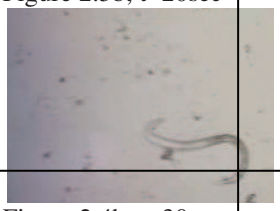

Figure 2.4b, t=30sec

Box c

Figure 2.1c, t=0sec

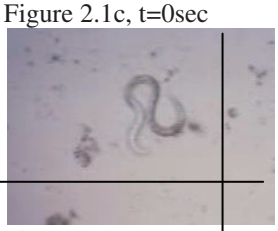

Figure 2.2c, t=10sec

Figure 2.3c, t=20sec

Figure 2.4c, t=30sec

*Note that the grid lines show that there is a significant movement of J2 without Pp spores compared to those encumbered with Pp spores Figs 1-4b and 1-4c.

Fig. 2. Nematode body wave formation presented as a rectangle shape movement over time. J2 were encumbered with Pp spores column b (low Pp) and c (high Pp) or without Pp spores column a. Arrows indicate the J2 speed.

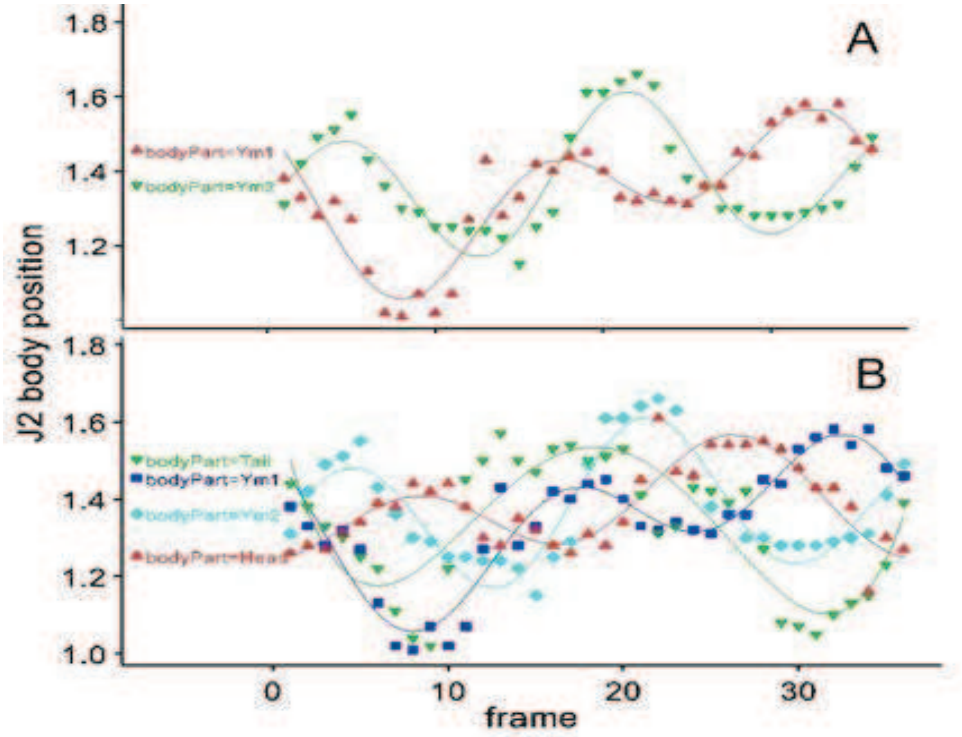

Fig. 3. Motion analysis and position of nematode body parts over time. Nematodes were not encumbered with *P. penetrans* endospores. Analysis was performed using GenStat Statistical Package based to (a) Ym1 and Ym2 data sets and (b) to four nematode tracking points (head, esophagus, gut and tail). *Y* axis, in promotion to nematode body length which is equal (in this study) to 1.8. and *X* axis, in frames where 39 frames are equal to 30 sec.

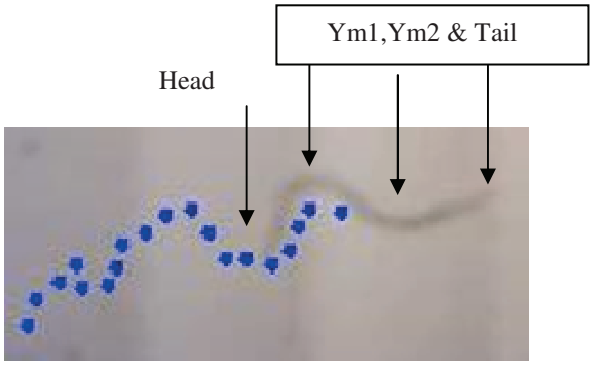

Fig. 4. Motion analysis (4a) and position (4b) of nematode body parts (XY) over time. Nematodes were free *P. penetrans* spores. Analysis was performed based to the Head, Ym1, Ym2 and to the J2 tail data sets. R^2 = 74.9, P<0.001. Axis scales x, y are measured in promotion to nematode body length which is equal (in this study) to 2.0.

The observation that a nematode body produces a sine wave is further tested using the MotionPro software program ver. 4.4.2., by PDSofTec (www.pdsoftec.com), based on head region turns. The directions of head region rotation were observed based on nematode movement and displayed as a set of points (dots) in real time when nematodes moved (Figure 4).

With nematodes free of spores we observed that the 2nd, 3rd and 4th body part follows the head movement. This does not happen when the nematode is encumbered with *P. penetrans* spores at low or high density (Figures 2.1b-2.4b and Figures 2.1c-2.4c).

3.2.1 Tracking the nematode rectangular shape area

The analysis showed that the nematode without *P. penetrans* spores produced a rectangular shape with a significantly greater area compared to nematodes encumbered with spores (Figure 5). This was confirmed with the Movie Maker 2 software program where each nematode video was observed (Figure 2). We conclude that nematodes without *P. penetrans* spores fit a rectangle shape with a strong forward movement equal to the rectangle shape in Figure 2 marked as box a, whereas nematodes encumbered at a low or high density of spores could fit to rectangles equal to boxes b and c in Figure 2. There was little movement of J2 encumbered with high *P. penetrans* attachment and several times they were seen to collide.

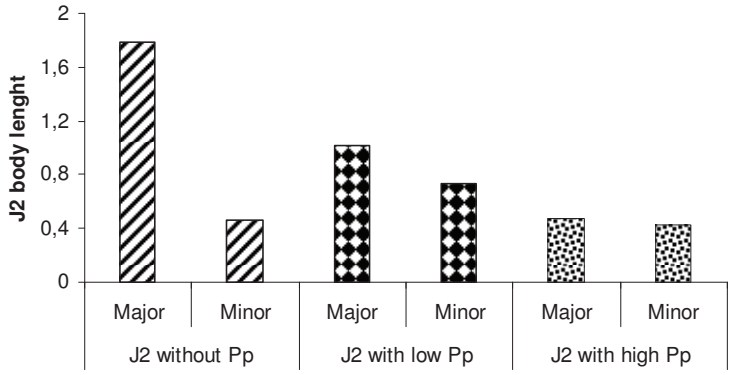

Fig. 5. Rectangle estimation results for J2 motion treated with (left) or without *P. penetrans* spores (middle and right). The major (long) rectangular side presents J2 forward movement where the minor (short) presents the width of the sinusoidal J2 body motion. The value of two (2) in *Y* axis is equal to a value for a straight J2 body length.

3.2.2 An estimation of J2 motility

The measurements based on the nematodes locomotion shows a significantly greater wavelength (λ) and distance movement values for nematodes without spores attached compared with nematodes encumbered at a low or high density of spores (Figures 6 and 7 respectively). Nematodes encumbered at a high spore density showed insignificant movement (Figure 7) confirming observations shown in Figure 2 where

nematodes' faraway body segments can be fitted in a box with an insignificant no forward movement.

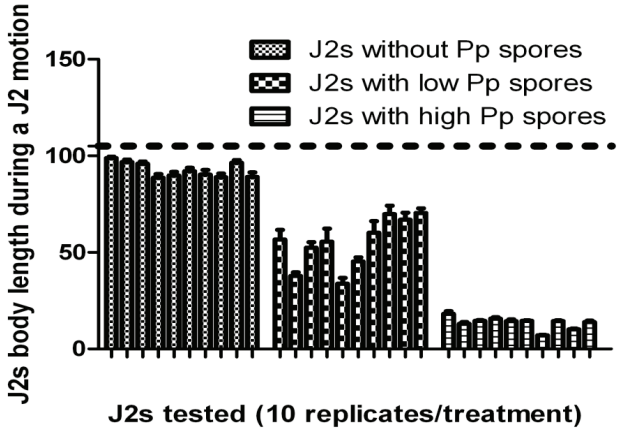

Fig. 6. Differences in J2 body length [= a nematode wavelength (λ)] during motion in treatments with or without *P. penetrans* spores. Dotted line represents a straight J2 body length which is equal (in this study) to the value 105 in *Y* axis.

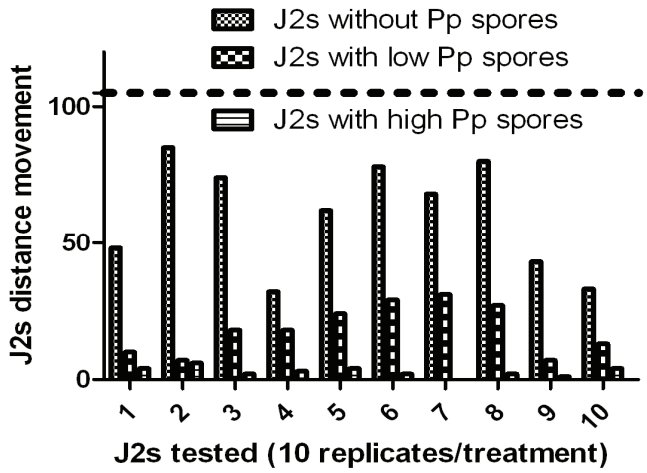

Fig. 7. Distance moved by J2 with or without *P. penetrans* spores (N=10) in 15 sequential frames (=11.5 sec). Dotted line represents a straight J2 body length which is equal to the value 105 in *Y* axis.

3 Discussion

In this paper we present a technique to track a plant parasitic nematode movement using a digital camera and an inverted microscope. Similar techniques where shown in Baek *et al.* (2002) and Cronic *et al.* (2005) where the authors used a microscope fitted with a camera and a videtaping (VCR) system to record movement of *Caenorhabditis elegans*.

Using free Internet software packages such as the SC Video Decompiler we extracted images (frames) from video files (.avi format) for further analysis. This is similar to Cronic *et al*. (2005) who took their data to a PC using a Matrox Meteor-II/Standard video frame grabber hardware and the Recognizer 2.1 software package.

Further, with the commercial software package Scion Image we collected data taking selected nematode body points. Similarly, Cronic *et al.* (2005) presented the same ideas for data extraction and processing in Matlab called Wormproc; they showed many *C. elegans* measurements and histograms grabbed at 13 points and from the centroid point of the body respectively. Greng *et al*. (2004) identified and tracked separately the head and tail movement of *C. elegans*. In our studies we employed the GenStat statistical program and the Standard Curve Routine, a tool of the regression analysis and we described effectively with the double Fourier curve the motion of four or two body points in nematodes without Pp endospores. Wallace (1958; 1959), Baek *et al*. (2002); Cronic *et al.* (2005) concluded that nematodes produce a sinusoidal movement. In our studies we showed that the nematode motion is a compound of two sine waves, one having half the cyclic period of the other (double Fourier curve).

A natural characteristic is that all nematode body parts follow the movement of the nematode head (Niebur and Erdos, 1991) and this is demonstrated. However this did not occur when the nematodes were encumbered with Pp endospores, probably because the spores impeded forward movement. Moreover we show that J2 encumbered with high numbers of sporesshow no forward movement and several times were observed to collide with other nematodes.

The nematode rectangular shape area produced by a nematode's body parts proved a very good estimator to describe nematode locomotion for nematodes without spores and those encumbered with low and high numbers.

Nematodes without spores move faster than those that are encumbered. Moreover those nematodes moved in a straight line in the same direction and covered a longer distance than those with endospores. Those nematodes pulse the body with a wavelength (λ) equal to a straight body position, probably this is a random walk with memory as suggested by Hapca *et al.* (2007). Wallace (1958) observed the same for *Heterodera schachtii* migration in soil and he concluded that the maximum speed of the *H. schachtii* J2 is attained when there is no lateral movement and each part of the body follows the part immediately in front of it.

Finally it can be suggested that this research could be improved with more emphasis on mathematics developing codes e.g. on the Matlab software package. Further the methods (the technique to track a plant parasitic nematode movement using a digital camera and a microscope) could be improved in wider range.

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