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SHORT NOTE

Adhering *Pasteuria penetrans* endospores affect movements of root-knot nematode juveniles

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Summary. *Pasteuria penetrans* is a biological control agent of root-knot nematodes (*Meloidogyne* spp.), preventing root invasion by second-stage juveniles (J2s), and eventually causing females sterility and death. Greatest control effects for *P. penetrans* depend on the numbers of endospores attached to nematode cuticles. A method based on digital image analysis was used to record the effects of endospore attachment on the movements of juvenile root-knot nematodes, using a model based on the centroid point. Data showed that the numbers of endospores attached to the cuticle influenced nematode movement. At high endospore attachment levels (20–30 per J2), nematodes did not show directional movement, whereas nematodes encumbered with five to eight spores showed limited directional movement, compared to those without endospores. Nematode cephalic region turns were modelled using a Markov chain, showing that *P. penetrans* endospores affected movements. Less nematodes invaded and established on tomato root systems when encumbered with low (five to eight) or high numbers (20–30) of *P. penetrans* endospores, compared with unencumbered nematodes.

Key words: modelling, Markov chain, biocontrol.

Introduction

Pasteuria penetrans is an obligate parasite of root-knot nematodes (*Meloidogyne* spp.). The bacterium is cosmopolitan, frequently encountered in different climates and environmental conditions, making it a promising candidate for the biological control of root-knot nematodes (Sayre and Starr, 1988; Hewlett and Dickson, 1994; Gowen *et al.*, 2008).

Successful parasitism depends on the attachment of five to ten spores per second-stage juvenile (J2), which is sufficient to initiate infection, without reducing the ability of nematodes to invade roots (Davies *et al.*, 1988; 1991; Rao *et al.*, 1997). It was observed that little or no root invasion occured when more than 15 endospores were attached to the host, inferring that attachment levels affect the ability J2s to locate and/or invade roots (Davies *et al.*, 1988). Few attempts have been made, however, to quantify the effect of *P. penetrans* endospore attachment rates on the movement of infective J2s.

Nematodes move by undulations or wave-like motions through dorsal/ventral contractions of the body (Storer *et al.*, 1979; Buchsbaum *et al.*, 1987). As one segment of the body contracts, the remainder of the body is "pulled" forward.

In the present study, a method was applied to track and record nematode movements using a microscope and a digital camera. The tracks of the J2s were analyzed with an image processing program (Image J). A further aim was to examine how *P. penetrans* endospores affect the motion of J2s toward a

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plant root and how a large number of endospores adhering to the cuticle can block motion into soil. A Markov chain model (Hoppensteadt, 1982; Choo *et al.*, 2004; Berresford and Rockett, 2004; Waner and Costenoble, 2011) was also used to describe the J2 movement process, with or without adhering *P. penetrans* endospores.

Materials and methods

Nematode cultures

Root-knot nematodes (*Meloidogyne javanica*) cultures were maintained on tomato plants (cv. Tiny Tim) in a glasshouse, and fresh J2s were collected from infected tomato roots using the method described by Hussey and Barker (1973).

Preparation of endospores

A commercial product based on a *P. penetrans* strain (Pp) (Nematech Co. Ltd., Japan) was used in this study. Fresh J2s were encumbered with Pp endospores as described by Darban *et al.*, (2004). Nematodes with five to eight endospores attached were considered as Low-Pp and J2s with 20–30 spores were the High-Pp treatments, respectively.

Acquisition of video images

Cohorts of J2s encumbered with five to eight or 20–30 endospores, or without endospores, were tracked with an inverted microscope (MICROTEC 200) mounted with a digital camera (Aiptek 3M). In all cases, a nematode's movement was observed in water in a 9 cm Petri dish. The microscope magnification was ×100 and the digital camera captured image frames with 320×240 pixels (3.3×2.5 inches). After recording, video sequences showing movements of an individual J2 were observed on Movie Maker 2 (Microsoft). Twenty random individuals from each treatment were selected for further analysis.

Image extraction

Before analysis, selected 40 sec videos were captured by means of a video decompiler (SC Video Decompiler, http://www.softwareclub.ws/v_decompiler.html) to extract frames, producing a total of 400 frames from a 40 sec video, which were then saved in .jpg format.

Image processing and analysis

Metrics of nematode movements were measured using image analyzer software Image J for Windows (http://rsb.info.nih.gov/ij). All images (frames) were saved in .tiff format before importing to the Image J software package. Images were ranked and every 50th frame was selected up to the maximum of 400 frames. When an image file (*.tiff) was imported to the Image J software program, measurements were performed with the "manual area measurement" selecting the "Measure" command. Moreover, in order to understand the aspects of the nematode body posture and speed, measurements of the J2 body movements were obtained from each frame tracking the centroid point of the nematode body. In detail, the measurements of the J2s with or without Pp endospores were the following.

Nematode distance travelled

Using the rectangular selection tool of Image J and moving the mouse on the nematode image, all faraway nematode body segments were fitted in a rectangular shape in order to estimate the X-Y geometric center (using the measurement "Options", "Area", "X-Y Center"). The geometric center (X-Y center of the rectangle area) was used to track the centroid point of each nematode body. Further measurement data of J2s with and without Pp endospores were extracted to an Excel® spreadsheet for analysis with GenStat (7th Edition, http://www.vsni.co.uk/software/genstat).

NemaTode cephalic region position

A Markov chain was used to model nematode cephalic region positions over a fixed period of time, recording the cephalic region rotation as left-right (LR), right-left (RL), right-right (RR) or left-left (LL). To display the cephalic region position, 25 random nematodes were observed, for both treatments (J2s without endospores or with five to eight endospores). Observations were made over 20 observed frames (t1 = 1 sec, t2 = 2 sec and t20 = 20 sec).

For Markov chain time, four dimensions were used (t1 to t2, t6 to t7, t13 to t14 or t19 to t20), allowing the construction of a Markov chain transition probability matrix P, for unencumbered J2s and a transition probability matrix Q, for encumbered J2s. The data were entered to an Excel spreadsheet to determine the transition probabilities of each transition matrix P and Q as follows:

	State form	(LL)	(LR)	(RL)	(RR)
	t1, t2	0.04	0.52	0.44	0
P =	t6, t7	0	0.36	0.48	0.16
	t13, t14	0.04	0.48	0.44	0.04
	t19, t20	0.08	0.24	0.68	0

	State form	(LL)	(LR)	(RL)	(RR)
	t1, t2	0.16	0.28	0.36	0.2
Q =	t6, t7	0.32	0.08	0.16	0.44
	t13, t14	0.2	0.32	0.2	0.28
	t19, t20	0.28	0.12	0.12	0.48

Each transition matrix (P and Q) was analyzed using a TI-83 Plus Graphing Calculator (Texas Instruments, USA). For greater accuracy, the output probabilities of states P^{256} and Q^{256} were computed.

Estimation of J2 motility

The movement of nematodes treated with low and high Pp endospore numbers, or without endospores were estimates based on the J2 length and the distance moved over time. The body lengths of each nematode were measured using the Straight Line Selections Tool (counts objects of the Image J program), moving the mouse on the nematode images to mark the cephalic and tail regions each time.. The same procedures were used to measure the distance travelled by the nematode cephalic region over time t1i and t2i (frames 1i and 2i) moving the mouse from X1i, Y1i (frame 1) to X2i, Y2i position on frame 2. Data were exported to an Excel spreadsheet as above. The absolute body length of a J2 was equal to a 105 values (in pixels) (Figure 3). Measurements were based on ten nematodes per treatment with 15 frames for each J2. The time between two frames in the sequence was 11.5 sec.

Effects of *Pasteuria penetrans* endospore attachment rate, on penetration and parasitism of root-knot nematodes *in planta*

Fresh J2s were encumbered with Pp endospores as described above, marking J2s with five to eight Pp spores per nematode as Low Pp and those with 20–30 Pp endospores as High Pp. Tomato plants, 4 weeks old, were inoculated with 300 ± 32 J2s/plant. Treatments were: plants without nematodes and endospores, plants with nematodes, plants with Low Pp nematodes and plants with High Pp. Treatments were replicated 16 times. Plants were maintained in a glasshouse at 26°C and after 25 days they were uprooted, washed under tap water and the number of females, root galls and nematode egg masses per plant were recorded (Table 1).

Results and discussion

Image processing and analysis

Nematode distance travelled

Over similar time periods the J2s free of *P. penetrans* endospores travelled further than those with *P. penetrans* propagules attached (Figure 1). Movements of J2s free of *P. penetrans* started from the low-

Table 1. Means of *Meloidogyne javanica* plant infection parameters for tomato plants inoculated with J2 nematodes treated with different amounts of *Pasteuria penetrans* (Pp) endospores.

Treatment	Females/ plant (mean ± SD)	Galls/plant (mean ± SD)	Egg masses/ plant (mean ± SD)
Control (J2s without Pp)	84.8 c ± 14.7	23.8 c ± 10.3	17.3 c ± 11.1
J2s with 5–8 Pp	45.5 b ± 15.8	10.5b ± 5.6	7. 5 ab ± 5.2
J2s with 20–30 Pp	17.4 a ± 4.0	1.8a ± 1.6	0.1 a ± 0.4
P value	P<0.001	P<0.001	P<0.001

Values within a column followed by the same later do not different significantly according to Tukey's tests (P=0.05). Values are based on 16 replicates per treatment.

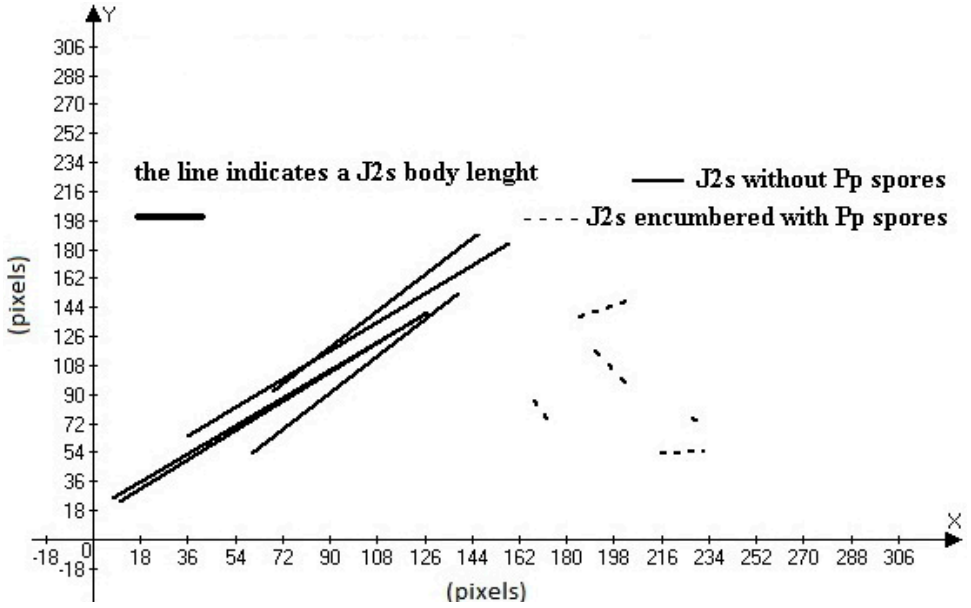

Figure 1. Movement of second-stage *Meloidogyne javanica* juveniles (J2s) with and without *Pasteuria penetrans* attached endospores. Each line represents the movement of a single J2. Each position was estimated by joining positions of centroid body points, as the nematode moved.

est point of the XY axis and ended at the extreme of the same axis. On the contrary, the movements of the nematodes encumbered with endospores appeared disorganised (Figure 1).

Nematode cephalic region position

The observed vs output (predicted) probabilities of the number of state P^{256} and state Q^{256} are shown in Figure 2A. The output probability P^{256} (predicted data, Figure 2A) shows a high probability of J2s turning in different direction and a low probability of turning in the same direction, demonstrating that transition matrix P^{256} captures the same properties of the initial state P^1 (Figure 2), suggesting that unencumbered J2s have a high probability of going the opposite direction. Moreover, Figure 2B captures the same properties of the initial state Q^1, where the output probability Q^{256} (predicted data, Figure 2B), shows a high probability of J2s turning in the same direction. Further, based on results obtained from the steady state distribution P^{256} and Q^{256}, the observed data of P and Q were fitted (Figure 2).

J2 motility

The measurements based on the nematodes movement show significantly greater distance movement values for unencumbered nematodes, compared with nematodes with low or high numbers of attached *P. Penetrans* (Figure 3). Nematodes encumbered at a high and low endospore densities (20–30 vs five to eight Pp spores) showed insignificant movements (Figure 3), in agreement with observation shown in Figure 1. Furthermore, the recorded data showed that movements of nematodes free of endospores were characterized by distribution with long steps followed by short steps (Figure 3).

Effects of *Pasteuria penetrans* endospore attachment rate on penetration and parasitism of root-knot nematodes *in planta*

The evaluation of *P. penetrans in planta* resulted in a lower rate of nematode invasion, penetration and development in tomato roots compared to controls (Table 1). The numbers of root galls and egg masses were significantly less in treatments with J2s encumbered with 20–30 *P. penetrans* (Table 1.) Results indicated that the bacterium exerted a "nematostatic effect" when J2s were encumbered with high numbers of endospores.

Techniques tracking a nematode movement with a digital camera and an inverted miscroscope were ap-

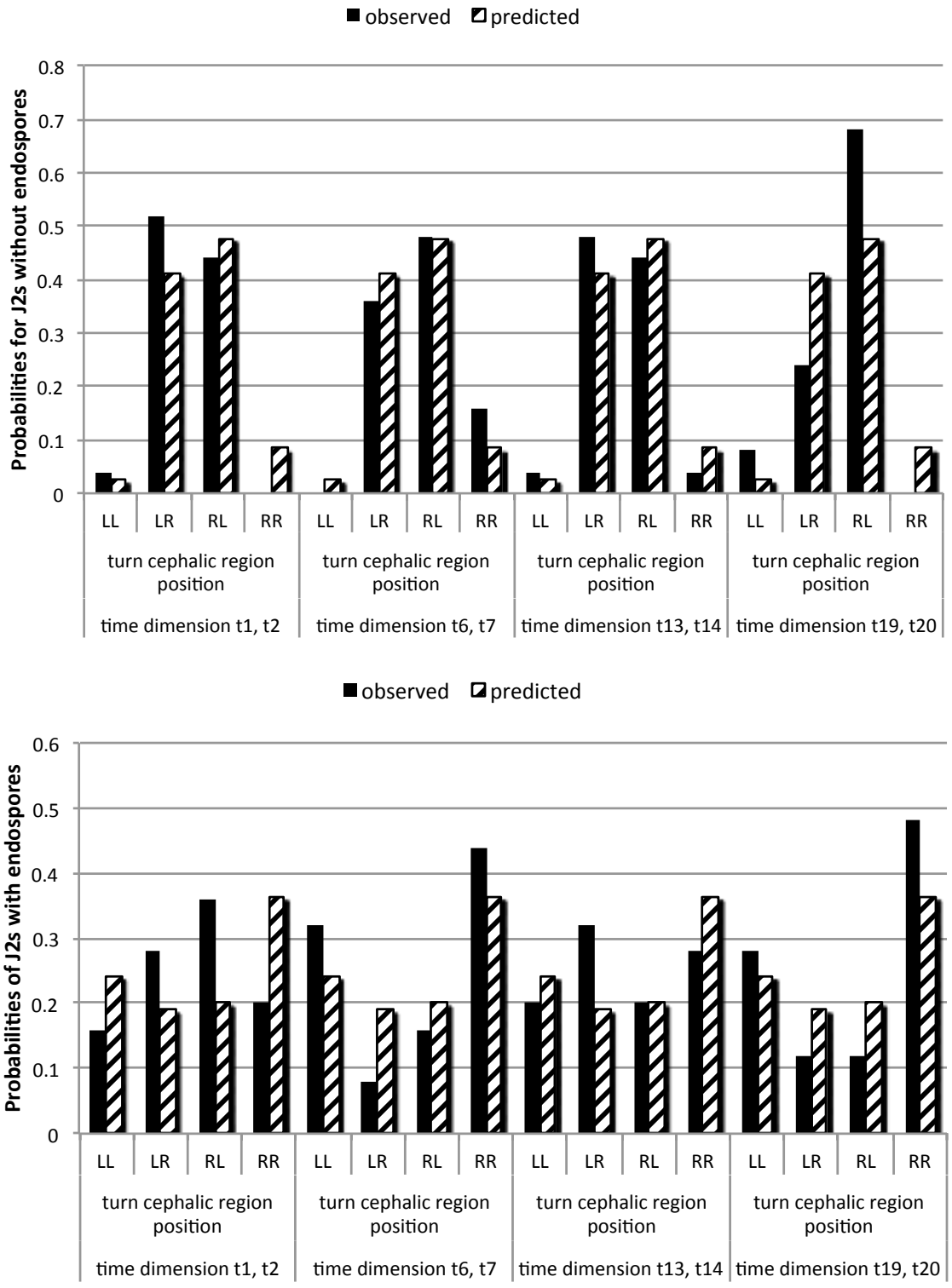

Figure 2. Observed and expected probabilities (at a fixed matrix P^{256}), of unencumbered second-stage *Meloidogyne javanica* juveniles (J2s) turning in the same or different directions (A), and predicted probabilities (at a fixed matrix Q^{256}), of J2s encumbered with *Pasteuria penetrans* endospores, turning in the same or different directions (B).

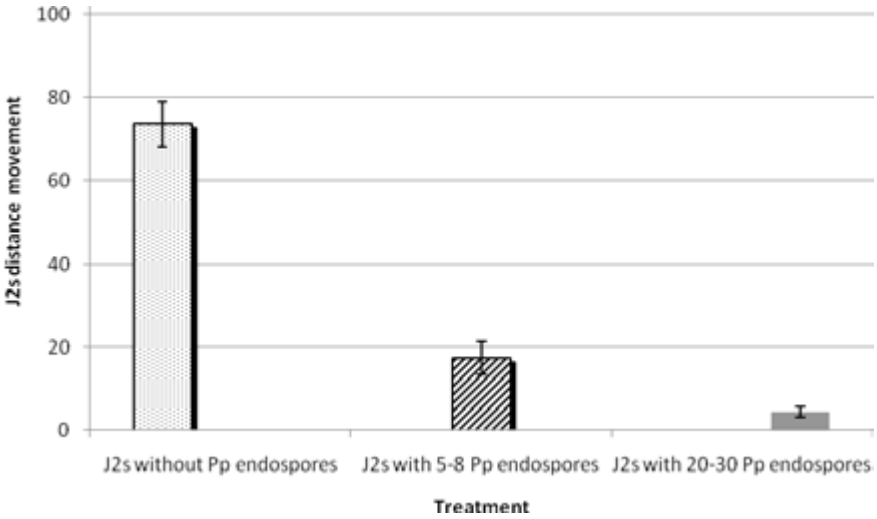

Figure 3. Differences in tracks of second-stage *Meloidogyne javanica* juveniles (J2s) with or without *Pasteuria penetrans* endospores observed *in vitro*. Observations were made over 15 observed frames at 11.5 sec intervals. Dotted line represents a straight J2 body length which is equal to the value 105 in *Y* axis.

plied by Baek *et al.*, (2002) and Cronic *et al.*, (2005), who used microscopes fitted with cameras and videotaping systems to record movements of *Caenorhabditis elegans*.

Data showed that nematodes free of endospores moved more quickly than those encumbered. Moreover, they moved in straight lines in one direction (Figure 1) and covered greater longer distances than those with endospores. Wallace (1958) observed the same for *Heterodera schachtii* migration in soil and concluded that the maximum speed of J2s was attained when there was no lateral movement and each part of the nematode body followed the part immediately in front of it.

Wallace (1958; 1959), Baek *et al*., (2002) and Cronic *et al.*, (2005) concluded that nematodes produce sinusoidal movements. In the present study, the motion of J2s appeared disorganized by the Pp endospores, indicating that high numbers of propagules attached to the nematode cuticles had significant impacts on movement (Vagelas *et al.*, 2011).

In a natural habitat, the J2 body parts usually follow the movement of the cephalic region (Vagelas *et al.*, 2011). However, this behavior was not observed in the present study when the nematodes were encumbered with endospores. The propagules appeared to interfere with the nematode forward movements and disorganized the nematode's labial region turns, as shown by others studies (Stirling, 1984; Davies *et al.*, 1994; Davies and Redden, 1997; Trudgill *et al.*, 2000; Davies, 2004; Wishart *et al.*, 2004; Davies, 2009).

The Markov model was an easy computation tool to study nematode motion processes. Several authors proposed the use of Markov chains as an efficient statistical test for application in biological modeling, where future outcomes can be predicted from observed counts (Leslie, 1945; Leslie, 1948; Nisbet and Gurney, 1982; Taylor and Karlin, 1998, Daley and Gani, 1999; Kot, 2001). However, further data are required to estimate effects of other factors on *P. penetrans*, including soil properties.

In conclusion, the bacterium at low attachment rates reduced and changed J2 movements, whereas at higher levels it halted directional movements. These results agree with the significantly low galling and egg mass numbers observed after one nematode generation (Table 1), when *P. penetrans* was present.

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