Effect of humidity and a superabsorbent polymer formulation on the efficacy of Heterorhabditis zealandica (Rhabditida: Heterorhabditidae) to control codling moth, Cydia pomonella (L.) (Lepidoptera: Tortricidae)

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Abstract
Adequate moisture levels are required for nematode survival and subsequent efficacy as entomopathogens. Formulation of nematodes aimed at aboveground applications may assist in maintaining such moisture levels. In this study, we report the effects of a superabsorbent polymer formulation, Zeba® on the performance of an entomopathogenic nematode, *Heterorhabditis zealandica* Poinar, for controlling diapausing codling moth, *Cydia pomonella* (L.) larvae in cryptic habitats on trees. Water activity (a_w-value) on bark was considered to be an indication of moisture levels on trees in cryptic habitats where codling moth larvae are known to occur, thereby influencing nematode efficacy. *H. zealandica* was only able to infect codling moth larvae at $a_w \geq 0.92$, with $a_{w50}=0.94$ and $a_{w90}=0.96$.

Laboratory experiments in which nematode concentration was investigated indicated a positive linear relationship between the concentration of nematodes applied and the level of control obtained, with the highest level of mortality recorded at 80 IJs/larva, requiring at least 4 h of conditions conducive to nematode activity to ensure infectivity and subsequent efficacy. Further experimentation showed that the use of the Zeba formulation, together with the nematodes, improved the level of control obtained at 60% and 80% RH in the laboratory and that it also enhanced the survival and infection-ability of the nematodes in the field. The study conclusively illustrates that the tested formulation assisted in maintaining adequate moisture levels on the application substratum, as required for nematode survival and subsequent efficacy.

Introduction
Codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), is a devastating pest of apples and pears throughout temperate regions of the world (Barnes 1991). Adult moths emerge from pupae in late spring and lay their eggs individually on the fruit and adjacent leaves. Neonate larvae hatch from these eggs and move towards the fruit, where they penetrate into the fruit to feed, creating a visible frass-filled tunnel. This action, together with its resultant appearance, reduces the market value of the crop (Welter 2008). Final instar larvae then exit from the fruit in search of a cryptic habitat, where they either cocoon and pupate or overwinter as diapausing larvae (Blomefield 2003). Depending on the temperature at which they are kept, codling moth may go through one to four generations per growing season (Barnes 1991).

Although entomopathogenic nematodes are generally pathogenic to a wide variety of insect pests (Poinar 1979), successful commercialisation and the use of the nematodes for biological control on a commercial basis have been growing from a limited to numerous insect pest on many different crops (Shapiro-Ilan, Gouge, and Koppenhöfer 2002; Sharma, Sharma, and Hussaini 2011). Codling moth has not only been proven to be susceptible to entomopathogenic nematodes in several research experiments (Lacey and Unruh 1998; Cossentine, Jensen, and Moyls 2002; Lacey, Neven, Headrick, and Fritts 2005; Lacey, 2006; Lacey, Arthurs, Unruh, Headrick, and Fritts 2006a, Lacey, Granatstein, Arthurs, Headrick, and Fritts 2006b; Lacey, Shapiro-Ilan, and Glenn 2010; Navaneethan, Strauch, Besse, Bonhomme, and Ehlers 2010), but it is also one of the few insect pests on which nematodes are currently used, on a commercial basis, as a control strategy in integrated pest management systems. Currently, commercially formulated quantities of nematodes are already available in Europe for the control of codling moth.

The developmental stage of codling moth that is best suited for control with nematodes is the diapausing larval stage, which occurs between late summer and early spring in such cryptic habitats as underneath loose pieces of bark or in pruning wounds on trees (Lacey and Unruh 1998; Navaneethan et al. 2010). Eliminating the stage concerned would protect fruit from damage during the following growing season (Lacey and Unruh 1998; Lacey, Arthurs, Knight, and Huber 2007).

Due to the aboveground location of diapausing codling moth larvae, the suggested time of application is during autumn or early spring, when the whole population of codling moth consist of diapausing larvae (Lacey et al. 2006a, 2010; Navaneethan et al. 2010) Low temperatures and low levels of relative humidity (RH) have been proven to be the most limiting factors in the use of these nematodes for the control of codling moth. In colder fruit production regions, cold-adapted species such as *Steinernema feltiae* could be applied, since their application has been proved to be effective at temperatures below 15°C (Grewal, Gaugler, and Wang 1996; Lacey and Unruh 1998).

In the Western Cape Province of South Africa, low levels of humidity during the day and low temperatures during the night, during the period of diapause, could be a problem for nematode application against codling moth. Genetically improved nematode isolates can partially help to overcome this problem (Mukuka et al. 2010), although manipulation of the habitat where the nematodes will be applied can offer a practical solution (Webster 1973). Improved nematode formulation, through the addition of certain hydroscopic additives such as gels and certain adjuvants, has been proven to allow for the partial prevention of the rapid desiccation of nematodes (Glazer 2002; Navaneethan et al. 2010; Lacey et al. 2010).

The overall objective of the present study was to contribute to the aforementioned research field by an evaluation of the superabsorbent polymer formulation known as Zeba® [starch-g-poly(2-propenamide-co-2-propenoic acid) potassium salt, Tongaat Hulett Starch] for improving the performance of the entomopathogenic nematode *Heterorhabditis zealandica* Poinar 1990 (SF 41) for the control of codling moth. The specific aims of the study were (1) to determine the required water activity level (a_w-value), (2) to determine optimal concentration, (3) to determine the exposure time required by the nematodes to cause satisfactory levels of larval mortality at different levels of humidity and (4) to further investigate whether the addition of the Zeba would improve the efficacy of the nematodes in semi-natural conditions in the laboratory and during field application.

Materials and methods

Nematodes and insects

Infective juveniles (IJs) of *H. zealandica* strain SF41, from the Stellenbosch University collection (Malan, Kguyen, and Addison 2006), were produced in *Galleria mellonella* (L.) larvae at room temperature. Harvested nematodes were stored horizontally in 150 ml filtered water in vented 500-ml culture flasks at 14°C and shaken weekly for aeration. One hour before commencing each experiment, IJ concentrations for all trials were quantified in the laboratory, using procedures described by Kaya and Stock (1997). Codling moth eggs and diet were obtained from the codling moth rearing facility, Entomon Technologies (Pty) Ltd., located in Stellenbosch, Western Cape, South Africa. From these eggs, larvae were reared on an artificial diet under diapausing conditions in growth chambers [photoperiod 10:14 (L:D), 25°C, 60% RH]. Fifth instar diapausing codling moth larvae collected were used for the experiments.

Bioassay protocol

To simulate semi-natural conditions, all experiments, except the water activity trial, were conducted with pear tree trunks 10 cm in length and with a circumference of approximately 17 cm. Trunks were obtained from a commercial pear orchard on the farm Timberley, located in the Stellenbosch district, Western Cape, South Africa. Trunks were kept at 45°C for 2 days to dry and eliminate other organisms. Six holes (1 cm

deep, 0.5 cm wide) were drilled into each trunk, and a diapausing codling moth larva was transferred to each hole. Larvae were allowed to spin up in cocoons inside the holes over a 24 h period prior to each experiment. For nematode application during the laboratory bioassays, an airbrush sprayer was used at 2 atm pressure. Nematodes were applied over the entire surface of each log at a concentration of 80 IJs/larva in 5 ml of water, except in the concentration experiment, where nematodes were applied at 0, 5, 10, 20, 40 and 80 IJs/larva. Controls were treated similarly but with water only.

To create the desired level of humidity, trunks were placed in sealed plastic containers, containing saturated salt solutions specific to each experiment. Containers were then incubated in climate chambers for 4 days at the start of a continuous temperature cycle of 10 h at 22°C, followed by 14 h at 11°C, to simulate average autumn temperatures in temperate regions. Temperature and humidity levels were monitored by Hobo® H8 Pro Series data loggers, which were placed inside the climate chambers. Four days after incubation, larvae were removed from the trunks, and their mortality was assessed.

Effect of water activity levels on codling moth mortality caused by *H. zealandica*

In order to assess the performance of *H. zealandica* (SF41) against diapausing codling moth larvae at reduced humidity, the isolate was tested at different water activity (a_w) levels. Adjusting and maintaining different a_w-values on pear tree trunks was impractical, so that the experiment was conducted in 3–cm-diameter Petri dishes lined with filter paper. Different volumes of water together with 50 IJs/larva suspended in each volume for treatments, or the same volume of water only in the case of controls, were added to each dish to create the required a_w-values, ranging from 6 to 28 µl as summarised in Table 1.

The a_w-values were measured using the Decagon Pawkit water activity meter (Decagon Devices, Inc., Pullman, WA, USA) at a constant temperature (25°C). Thereafter, five diapausing codling moth larvae were added to each dish and covered with cling wrap and a lid to ensure an airtight seal. Dishes were incubated at the optimum infectivity temperature of 25°C for 48 h, where after larval mortality was assessed. Five replicates were prepared for both treatment and control dishes for each a_w-level tested, and the experiment was repeated on two separate dates.

Influence of different nematode concentrations and RH on codling moth mortality

Using the trunk bioassay protocol, the effect of different nematode concentrations and levels of humidity on mortality was assessed. Saturated salt solutions in closed plastic containers (60×120×20 cm) were used to achieve 60% (glycerol), 80% (KNO_3) and 100% RH (containers lined with moistened tissue paper) (Winston and Bates 1960). Five trunks (each containing six diapausing codling moth larvae) were prepared for each of the different tested nematode concentrations (0, 5, 10, 20, 40 and 80 IJs/5 ml of water) at each of the different levels of humidity (60%, 80% and 100% RH), and the experiment was repeated on two separate dates.

Influence of exposure time on codling moth mortality

To ensure infectivity under ideal conditions, the influence of exposure time on larvicidal activity was assessed using the standard bioassay protocol to determine how long trees should be kept moist. Ten trunks (each containing six diapausing codling moth larvae) were prepared for each of the time intervals tested. Nematodes were applied to trunks at a concentration of 80 IJs/larva in 5 ml of water and incubated at 100% RH in sealed plastic containers lined with moistened filter paper. An equal number of trunks were also prepared for control treatments, with the same volume of water only being applied to each trunk.

After treatment and the subsequent incubation period, larvae were removed from trunks at 30, 60, 180, 240 and 480 min intervals, rinsed with filtered tap water to remove surface nematodes and placed in Petri dishes lined with moistened filter paper to allow nematode development for a further 3 days, after which their mortality was assessed. The experiment was repeated on two separate days.

Effect of a Zeba formulation in tree trunk laboratory bioassay

To test the superabsorbent polymer known as Zeba, together with the nematodes at a concentration of 3 g Zeba/L water, tests were conducted using pear tree trunks and following the standard bioassay procedure. The only alteration in procedure was the pre-wetting of logs to an a_w-value of 0.96 to simulate the general bark surface moisture of a live tree (Navaneethan et al. 2010). As described in the concentration experiment, saturated salt solutions in closed plastic containers were again used to achieve 60% and 80% RH for incubation.

Ten trunks, each containing six diapausing codling moth larvae, were prepared for each of the two treatments, which included (1) Zeba + nematodes and (2) nematodes only. For control treatments, an additional 10 trunks that received water only were prepared for each of the treatments concerned. Nematodes were again applied at a concentration of 80 IJs/larva in 5 ml of water. The trial was repeated on two separate dates and at two different levels of humidity (60% and 80% RH). Water activity (a_w-value) was also measured for each of the treatments at the start, and again at the end, of the trial period.

Field application

The effect of formulation on the efficacy of *H. zealandica* (SF 41) against diapausing codling moth larvae under field conditions was evaluated in a Forelle pear orchard on the experimental farm, Welgevallen, in the Stellenbosch district, Western Cape, South Africa. The field experiment was conducted mid-morning during winter on 14 June 2011. A completely randomised block design, with four rows, each containing eight treatment trees, with three buffer trees between each treated tree and two buffer rows separating treatment rows, was used for the experimental layout. As previously described in the standard bioassay protocol, pear tree trunks, each containing 10 diapausing moth larvae, were used for insect containment in the field experiment. Treatments were (1) nematodes, (2) nematodes + Zeba, (3) nematodes + Zeba + Nu-Film-P® (poly-1-p-menthene, spreader/sticker, Hygrotech) and (4) water, as a control treatment.

Nematodes were applied at a concentration of 50 IJs/cm^2, Zeba at 3 g/L water and Nu-film-P at 0.6 ml/L water. Treatment formulations were prepared on the day of the trial. Three trunks, containing codling moth larvae, were fastened onto the scaffold branches 1 m above ground of each of the 32 treatment trees on the day of the trial. Treatment trees and trunks were thoroughly pre-wetted (with 1 L water per tree) 1 h before treatment applications. Thereafter, 20 ml of treatment suspension was applied directly onto each trunk. All treatment applications were done using plastic calibrated handheld spray applicators at 2 atm pressure. After 24 h, the trunks were removed from the trees and taken back to the laboratory. Larvae were then removed from the trunks, washed to remove surface nematodes, placed in Petri dishes lined with moistened filter paper (Whatman No. 1) and incubated for a further 48 h at 25°C, whereupon larval mortality and nematode infection were assessed.

To evaluate the longevity of nematodes on trunks during the field trial period, five pieces of bark (2 cm^2) were removed from treatment trunks every hour for the first 8 h and rinsed in 50 ml filtered water. The resulting water sample was then transferred to 50-ml glass cylinders, and nematodes were allowed to settle at the bottom. A 5-ml volume of the resulting concentrate was examined under the microscope and the percentage of IJ survival was determined by movement response when probed with a dissecting needle (Kaya and Stock 1997; Shapiro-Ilan et al. 2010). Pieces of bark were also removed from treatment trunks hourly and placed in Petri dishes, to which 10 codling moth larvae were added, to determine infectivity at each of the different hourly intervals, for each of the nematode treatments. These dishes were kept at 25°C and infectivity was determined after 48 h.

Throughout the field trial period, weather data were downloaded from the Helderberg Weather Station in Stellenbosch, and Hobo H8 Pro Series data loggers were placed in the middle of each treatment row on a tree's scaffold branch to monitor temperature and humidity in the orchard environment.

Statistical analyses

All laboratory experiments were repeated on different test dates and the results combined for analysis. Abbott's formula was used to correct the data for natural mortality (Abbott 1925). All statistical analyses were performed using Statistica 9.0 software (Statsoft, Inc. 2009). Analysis of variance (ANOVA) was used to compare the different treatments, followed by Bonferroni multiple comparisons. When residuals were not normally distributed, bootstrap multiple comparisons were used (Efron and Tibshirani 1993). To evaluate the water activity experiment statistically, a Probit analysis was conducted using Polo PC (LeOra Software 1987). The aw_{50} and aw_{90} values were estimated with their 95% fiducial limits. All values throughout the text are given with corresponding standard errors.

Results

Effect of water activity levels on codling moth mortality caused by *H. zealandica*

All water activity (a_w) levels tested caused some degree of larval mortality, except a_w values < 0.92. Analysis of the results using a one-way ANOVA illustrated a significant positive effect of increasing water activity (a_w) on larvicidal activity (F = 46.55; df = 11,108; p<0.001) (Figure 1). Very low levels of mortality (≤15%) were recorded at a_w=0.8–0.93, where after mortality increased significantly from 15±10.67% at a_w=0.93 to 95±3.33% at a_w=0.95 (p<0.001). Mortality levels of ≥87% with no significant difference were observed at a_w=0.95–1.00 (p=1.00). The Probit analysis's regression equation for the combined data was Y = 5.12 + 126.94 [X], where Y = log (a_w). The a_{w50} and a_{w90} values and 95% fiducial limits were 0.94 (0.92–0.95) and 0.96 (0.95–1.00). No mortality of larvae was observed for the controls.

Influence of different nematode concentrations and RH on codling moth mortality

Mortality was below 30% for all the levels of concentration tested (data not presented) at both 60% and 80% RH and only the results obtained at 100% RH were therefore analysed, using a one-way ANOVA (F = 25.84; df = 5,54; p<0.001). A positive relationship between larvicidal activity and nematode concentration was determined from the results, with an incremental increase in mortality as nematode concentration increased (Figure 2). No mortality was observed when water only was applied. Relatively low levels of mortality (16%–58%) were observed at nematode concentrations of 5–40 IJs/larva. Satisfactory levels of larvicidal activity (90±6.61%) were only recorded at 80 IJs/larva.

Influence of exposure time on codling moth mortality

Larvicidal activity was recorded for each of the different exposure times (30–480 min) tested, with differences amongst treatments (F = 10.21; df = 4,95; p<0.001). Significantly higher levels of mortality (ranging between 47% and 53%) were obtained after exposure to the nematodes for ≥ 240 min, as opposed to a shorter exposure time (30–80 min) where mortality was below 25% (Figure 3) and no significant differences were observed (p<0.001). Results analysed with a one-way ANOVA, therefore, suggest that mortality significantly increases as exposure time lengthens to 240 min (p=0.002), where after no additive effect was observed (p=1.00).

Effect of a Zeba formulation in tree trunk laboratory bioassay

The two-way ANOVA (treatment×humidity) showed no interaction between the main effects (F = 0.20; df = 1,116; p=0.66). Treatments behaved consistently over the two levels of humidity tested, and main effects were therefore interpreted directly. The interpretation showed that the addition of Zeba significantly increased the efficacy of the nematodes at both levels of humidity tested. At 60% RH, mortality increased from 8±4.15% to 23±4.15% (p=0.07) and at 80% RH from 17±4.15% to 36±4.15% (p=0.01) (Figure 4).

Using a one-way ANOVA, the data for both levels of humidity were pooled for a further analysis to illustrate that the use of Zeba significantly increased the level of mortality obtained, from 12±2.94% to 29±2.94% (F = 16.32; df = 1,116; p<0.001). Water activity measurements also reflected the additive effect of using Zeba, as opposed to nematodes only, showing that water activity levels decreased for all treatments from a_w=1.00 directly post-treatment to a_w=0.93 (with Zeba) and a_w=0.89 (without Zeba) at 80% RH and a_w=0.70 (with Zeba) and a_w=0.54 (without Zeba) at 60% RH at the end of the trial period.

Field application

Moderate temperatures (ranging between 12°C and 30°C) were recorded throughout the trial period. The RH in the orchard was low (≈35% RH) at the time of application and increased after approximately 12 h due to light rain that was recorded inconsistently throughout the last 12 h of the trial period (Figure 5). Larval mortality recorded for the three trunks per tree were combined for the purpose of analysis, using a one-way ANOVA. Relatively equal levels of mortality due to nematodes were obtained for the three nematode treatments, as opposed to the control treatment where the level of natural mortality was significantly lower (F = 4.55; df = 3,28; p<0.01) (Figure 6).

Field results thus indicated that the addition of Zeba (T2) and Zeba + Nu-Film-P (T3) had no makeable effect on larvicidal activity in the field. For all three nematode treatments tested, nematode survival rate declined as the trial progressed (Figure 7). The average survival rate for the formulated nematode treatments was higher than for Treatment 1, where nematodes were applied with water only. The biggest decline in survival rate occurred during the first 3 h post-application, especially for Treatment 1. For all three nematode treatments, nematode infectivity also declined as the trial progressed (Figure 8). Infectivity was only recorded for the first 3 h for Treatment 1, as opposed to Treatments 2 and 3, where infectivity was recorded for up to 4 and 5 h, respectively.

Discussion

The literature indicates that *S. feltiae* and *Steinernema carpocapsae* are the most promising nematode species for the control of codling moth and therefore the most widely used (Kaya et al. 1984; Lacey and Chauvin 1999; Vega et al. 2000; Unruh and Lacey 2001; Cossentine et al. 2002; Lacey and Unruh 2005; Lacey et al. 2006a,b; Navaneethan et al. 2010). Although this is partially due to their commercial availability in most countries, it is also because of their biological characteristics (Grewal et al., 1996; Vega et al., 2000). In South Africa, however, neither of these two species has been isolated to date (Malan et al. 2006; De Waal 2008; Hatting, Stock, and Hazir 2009) and current legislation prohibits the import of exotic species (Agricultural Pest Act 36 of 1947).

As the study was conducted in South Africa, a locally obtained isolate of *H. zealandica* was used. This isolate previously proved to be relatively effective for the control of codling moth (De Waal 2008; De Waal, Malan, Levings, and Addison 2010; De Waal, Malan, and Addison 2011). The current study contributed to developing a science-based solution to further increase the efficacy of this isolate, by overcoming the issue of nematode desiccation prior to larval infection due to exposure to low levels of humidity, which, as was previously mentioned, is the biggest limiting factor for this type of application.

A clear distinction should be made between moisture-levels pertaining to the macro-environment (surrounding orchard humidity) and those pertaining to the micro-environment (the cryptic habitat where larvae, for example, reside underneath loose pieces of bark on the tree), as conditions may differ considerably between the two environments concerned (Navaneethan et al. 2010). Water activity (a_w-value) gives an indication of the amount of available water on the bark surfaces of trees, and thus of the effectiveness of nematodes in this type of micro-environment, as nematodes required a water film for propulsion (Koppenhöfer 2007). Navaneethan et al. (2010) documented the first investigation of the influence of water activity on nematode efficacy, using *S. feltiae*.

The current study elaborated on the concept, except that *H. zealandica* was used as a test isolate. Results indicated that larvicidal activity was still recorded down to a_w=0.92. To ensure a 90% mortality rate, the results indicated that an a_w-value of at least 0.96 should be maintained on trees during an application. The results obtained were similar to the findings for *S. feltiae* in the aforementioned study, where larvicidal activity was recorded down to 0.90 with a_{w90}=0.99 (Navaneethan et al. 2010). It has been noted that bark from living trees have an a_w-value of approximately 0.96 (Navaneethan et al. 2010), which, based on the results obtained, would theoretically support nematode activity and which would ensure infection to a relatively satisfactory level of control.

However, as suggested by the positive relationship between increasing water activity and the subsequent level of control observed from the results of the current study, even higher levels of control can be attained by the application of greater volumes of moisture. In addition, the findings of Lacey et al. (2006a) indicate that bark surfaces require to be wet for at least 8 h after nematode application and that a pre-wet could further enhance the efficacy of an application. The risk of over-irrigating trees does, however, exist, which would lead to drip and the subsequent loss of nematodes. Water is also a limited resource in most temperate regions, so that abiding by such a recommendation under such conditions could lead to incurring what would otherwise be avoidable costs.

Increasing the concentration of nematodes during an application has previously been shown to enhance the eventual level of control (Navaneethan et al. 2010). Similar findings were obtained from the current study, where a positive relationship was observed between the number of nematodes and the eventual level of control, with the highest level of control being obtained when using 80 IJs/larva. The correlative relationship between the variable cost factor involved in nematode production and the proposed increased amount of nematodes for enhanced efficacy should, however, be taken into consideration before practical implementation.

As was previously mentioned, the ambient humidity in the macro-environment also contributes to the success of an application. In the concentration experiment, it was illustrated that, where mortality was below 30% for all trials incubated at 60% and 80% RH, as opposed to 100% RH, satisfactory levels of control (up to 90%) were obtained. The results obtained were consistent with the findings of Lacey and Unruh (1998), where nematodes were only active at maximum levels of humidity (>95% RH).
Optimal humidity levels need to prevail long enough to ensure the successful penetration of the insect by the nematodes (Lacey et al. 2006a,b).

The exposure-time experiment showed that exposure of the larvae to the nematodes for half an hour was already sufficient for larvicidal activity. Prolonging exposure time thereafter further increased mortality. The same pattern was, however, only observed up to 4 h, where after no significant increase in mortality was recorded. This implies that the first 4 h post-application is the most crucial for ensuring successful larval infection. To ensure the efficacy of the treatment, trees should therefore ideally be kept wet for said period. It is, however, important to remember that other environmental factors such as wind and temperature could also influence the desiccation rate, and it is, therefore, advisable to maintain high moisture levels in an orchard for as long as possible post-treatment.

Previous exposure time trials conducted with *H. zealandica* showed that the degree of crypticness of larval location also influences the amount of time that is required for the nematodes to reach and infect the codling moth larvae. When nematodes were, for example, applied directly onto perforated cardboard strips containing codling moth larvae, only 5 h of high humidity were required to achieve 95% mortality, as opposed to in a mulch treatment, where larvae were hidden approximately 5 cm below the substratum surface, and almost 3 days of optimal conditions were required for the nematodes to locate and infect 95% of the population (De Waal 2008; De Waal et al. 2011).

Where pear tree trunks were used for insect containment in the current study, host contact was relatively assured. Larvae were allowed to spin their cocoons in holes that were drilled 1 cm into the trunks, and as the nematodes were applied directly onto the trunk surface, the only physical barrier through which the nematodes had to penetrate was the larval cocoons, which Navaneethan et al. (2010) showed not to be a limiting factor. The pear trunks used in this study did not contain enough bark for the nematodes naturally to hide and spin into cocoons. Drilled holes may also have been to the disadvantage of the nematodes, as the codling moth larvae bore more deeply into the wood, plugging the holes concerned with wood chewings.

In several recent publications, improved formulation has been noted to increase the efficacy of aboveground applications of nematodes for the control of certain orchard pests (Navaneethan et al. 2010; Lacey et al. 2010; Shapiro-Ilan et al. 2010). In the current study, similar results were obtained in the laboratory formulation experiment, whereby the addition of Zeba with the nematodes – as opposed to applying nematodes only – almost doubled the level of control obtained at 60% and 80% RH, and water activity levels on the bark also remained more consistent with the addition of Zeba, which therefore favoured survival and subsequent efficacy of the nematodes. Van Niekerk (2012) showed that, after 24 h exposure to Zeba, at the recommended dosage, no significant difference was observed over time when the treatment was compared to the control.

During the field experiment, moderate temperatures (ranging between 12°C and 30°C) during the field experiment would theoretically have ensured the survival of the nematodes (Koppenhöfer and Fuzy 2008). However, low levels of humidity (≈35% RH) during the first few hours after application would have been detrimental to the survival of the nematodes (Koppenhöfer and Fuzy 2008) and could partially explain the low levels of mortality obtained (≈55%) for all three nematode treatments. The non-significant difference in mortality obtained between the three nematode treatments containing either only nematodes or nematodes in formulation: Zeba or Zeba + Nu-film-P, would suggest that the formulation evidently had no probative effect on nematode efficacy. The results obtained were confusing, as the previous laboratory-formulation experiment indicated an evidential increase in mortality with the improved formulation.

However, the nematode survival and codling moth infectivity experiments confirmed the aforementioned expected results as a more gradual decline in nematode mortality was observed on bark pieces when nematodes were applied in formulation, as opposed to when nematodes only were applied, and a longer codling moth infection period was recorded for nematodes applied in formulation as opposed to for non-formulated nematodes. Regarding formulation (Zeba vs. Zeba + Nu-Film-P), results from both the nematode survival and infectivity experiments indicated that the use of both Zeba and Nu-Film-P yielded the best results and would, in future, possibly be recommended for the formulation of *H. zealandica* for the improved control of codling moth.

The study conclusively illustrated the required water activity levels in the micro-environment to ensure the survival and subsequent efficacy of *H. zealandica*, and it furthermore highlighted the importance of optimising other contributing factors, such as exposure time and nematode concentration. Although the field trail did not show that the addition of Zeba enhanced the efficacy of *H. zealandica* in the specific orchard environment under consideration, under laboratory conditions, it was shown that Zeba doubled the level of control and prolonged the longevity of the nematodes. Further field trials should be conducted to determine whether the positive effect of Zeba is advantageous in the more natural and optimum environmental conditions that prevail in the field for *H. zealandica* in order to ensure the successful use of these nematodes as part of an integrated pest management programme for the eventual control of codling moth in commercial deciduous fruit orchards.

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Figures

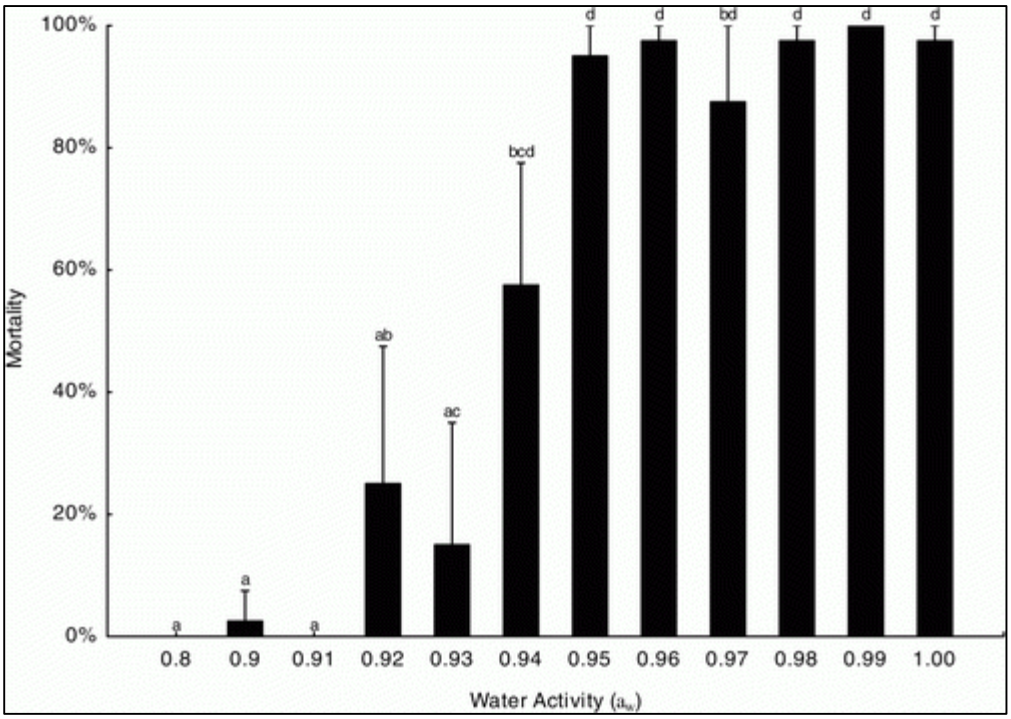

Figure 1. Mean percentage mortality (95% confidence interval) recorded for diapausing codling moth larvae after exposure to 50 infective juveniles per larva of *H. zealandica* (SF41) at different levels of water activity (a_w). Different lettering above vertical bars indicates significant differences (one-way ANOVA; $F = 46.55$; df = 11,108; $p<0.001$).

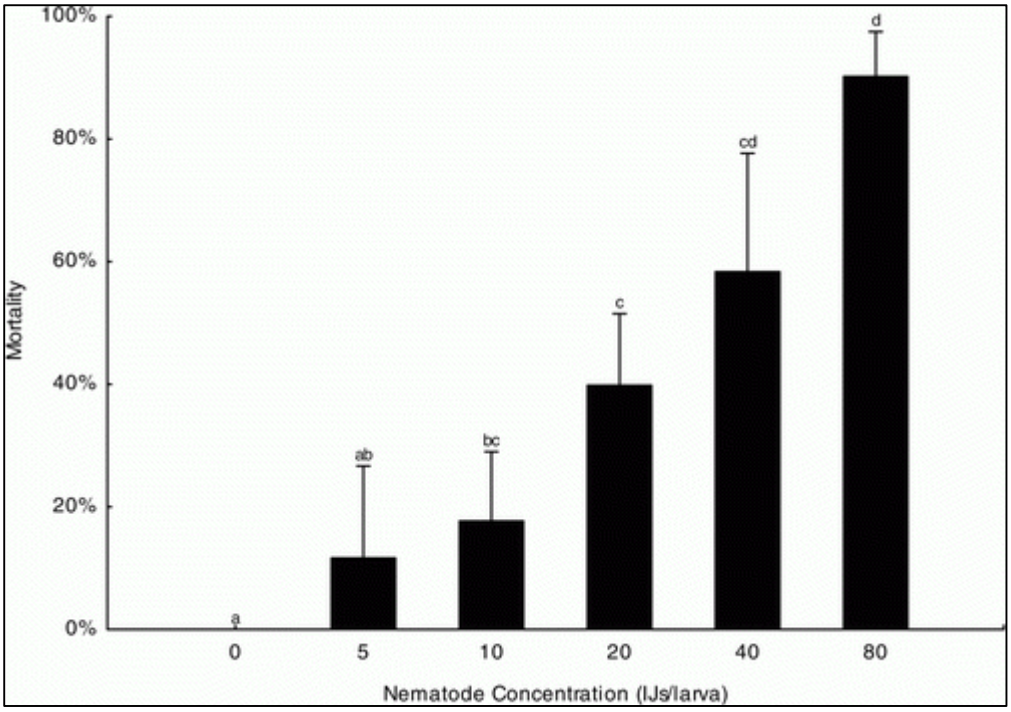

Figure 2. Mean percentage mortality (95% confidence interval) recorded for diapausing codling moth larvae after exposure to different concentrations (infective juveniles/larva) of *H. zealandica* (SF41) at 100% humidity. Different lettering above vertical bars indicate significant differences (one-way ANOVA; $F = 25.84$; df = 5,54; $p<0.001$).

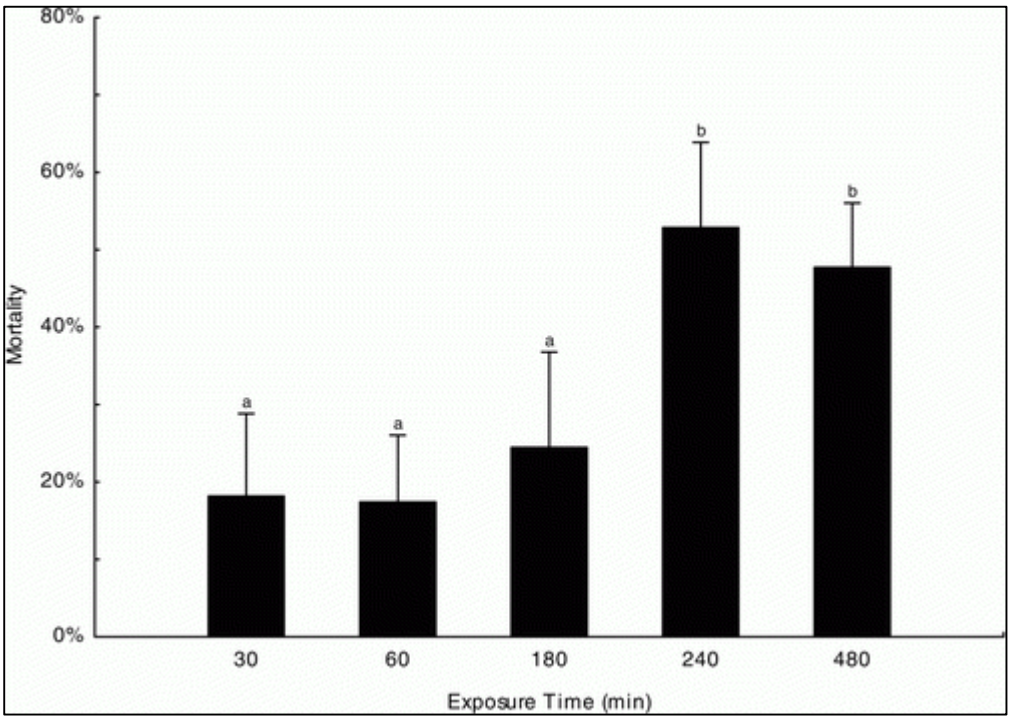

Figure 3. Mean percentage mortality (95% confidence interval) recorded for diapausing codling moth larvae after exposure to 50 infective juveniles/larva of *H. zealandica* (SF41) for different lengths of time at 100% RH. Different lettering above vertical bars indicate significant differences (one-way ANOVA; $F = 10.21$; df = 4,95; $p<0.001$).

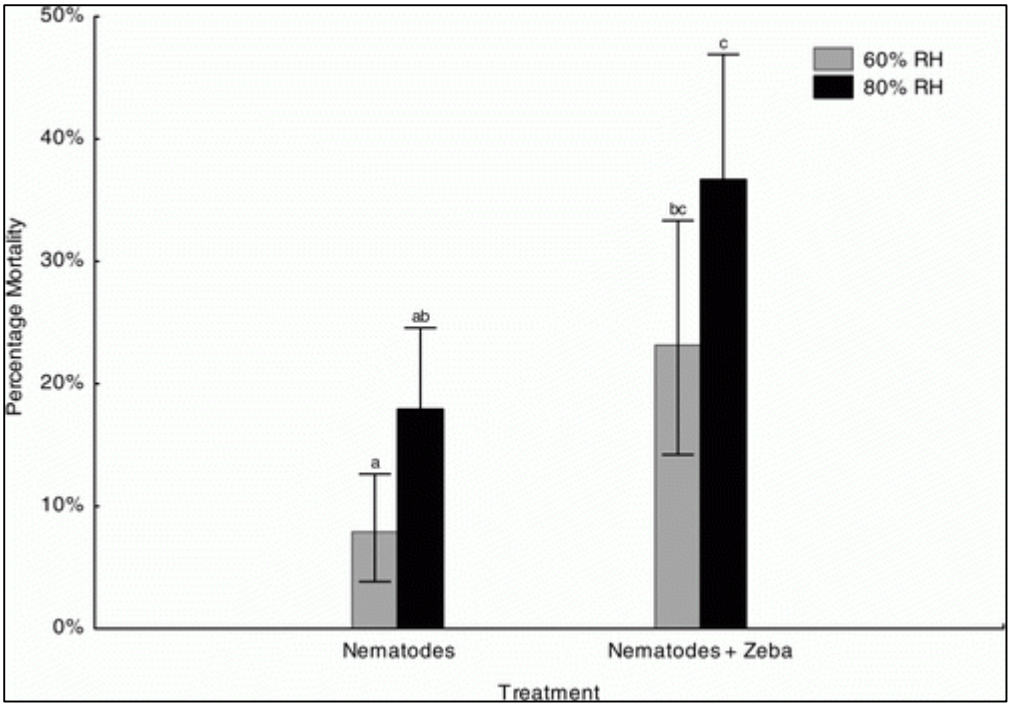

Figure 4. Mean percentage mortality (95% confidence interval) recorded for diapausing codling moth larvae after exposure to *H. zealandica* (SF41) with and without Zeba at 60% and 80% RH. Different lettering above vertical bars indicates significant differences (two-way ANOVA; $F = 0.20$; df = 1,116; p=0.66).

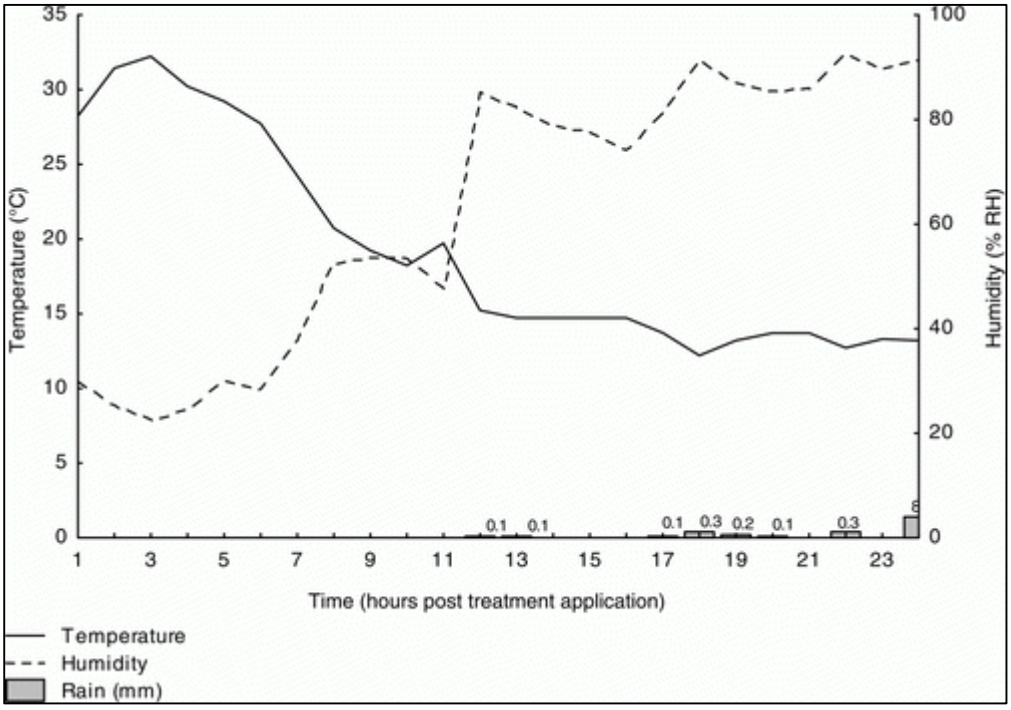

Figure 5. Climatic data recorded over a 24-h period during a field experiment.

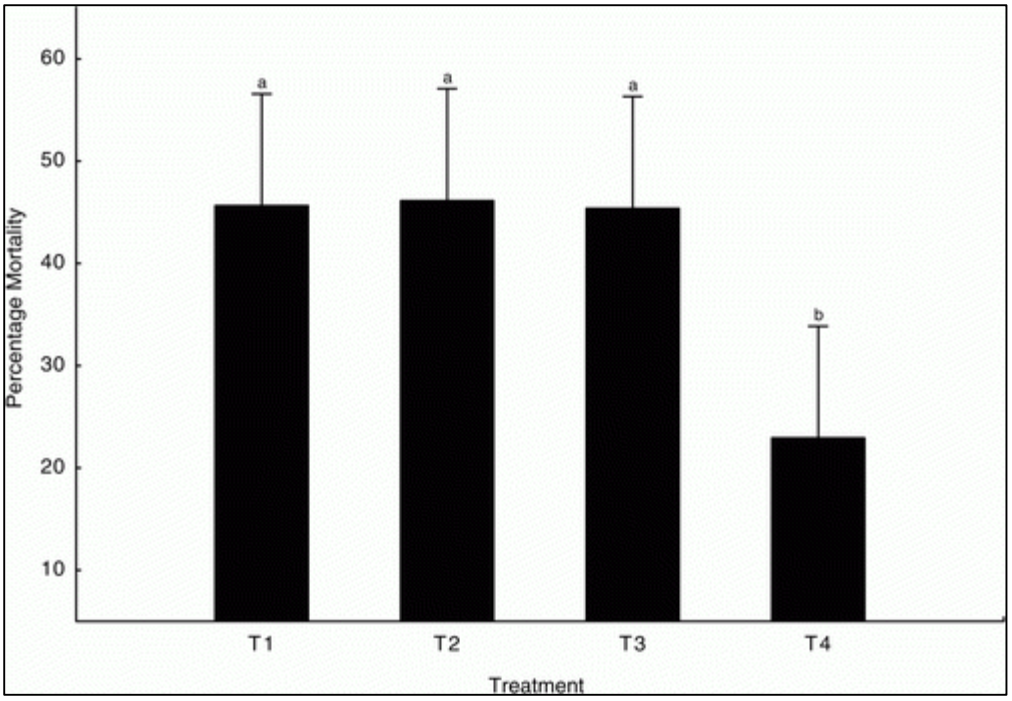

Figure 6. Mean percentage mortality (95% confidence interval) recorded for diapausing codling moth larvae after exposure to different formulations of *H. zealandica* (SF 41) during a field trial conducted in June 2011. Treatments were: (1) nematodes, (2) nematodes + Zeba, (3) nematodes + Zeba + Nu-Film-P and (4) water as a control treatment. Different lettering above vertical bars indicate significant differences (factorial ANOVA; $F = 4.55$; df = 3,28; $p<0.01$).

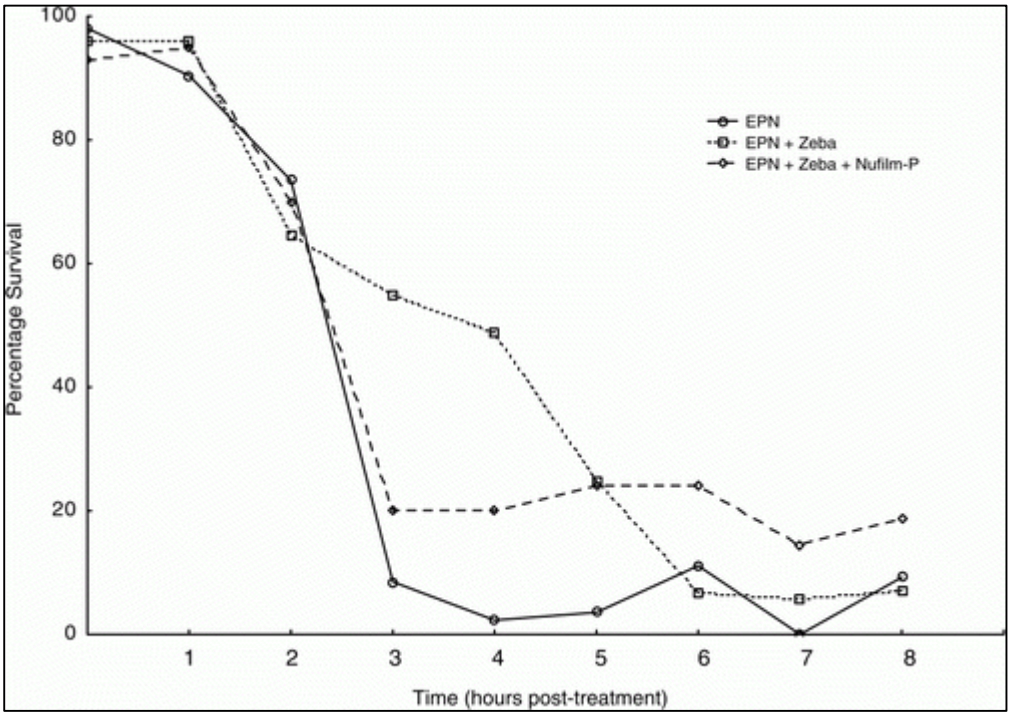

Figure 7. Percentage nematode survival recorded for *H. zealandica* (SF 41) on pieces of bark at different time intervals post-treatment.

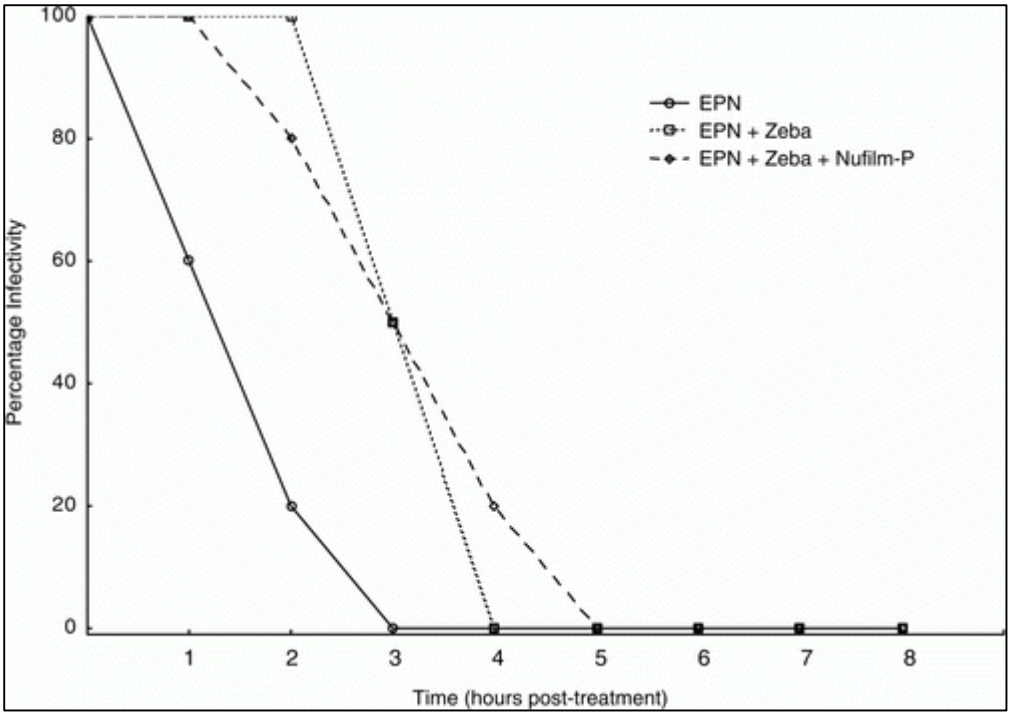

Figure 8. Percentage codling moth mortality recorded after exposure to *H. zealandica* (SF 41) for different time intervals during a field trial.

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