

ON THE WAY FOR A NEW BIONEMATICIDE

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Abstract

Pesticides, chemical synthesised products, are toxic compounds causing major environmental and human health problems, are unavoidably used to control plant enemies. Pine Wood Nematode (PWN), Bursaphelenchus xylophilus and Root-knot Nematodes (RKN), Meloidogyne spp., cause significant losses to pine forest and a wide range of plant species, respectively. The aim of this work was to study the effect of carvone on PWN and RKN. Carvone the main compound of the essential oil of caraway (Carum carvi L.) fruit is Generally Recognised As Safe and approved as Food Additive by the United States Food and Drug Administration. In vitro experiments were carried out in glass blocks. For both nematode species 100% mortality was registered in pure carvone and in carvone 2000 ppm concentration, 2 and 18 hours after exposure, respectively. The nematocidal effects of carvone on these nematodes suggest that this phytochemical may be a promising alternative to chemical synthetic pesticides.

Key words: biocide, bursaphelenchus xylophilus, carvone, meloidogyne javanica, root-knot nematode, pine wood nematode

1. INTRODUCTION

Why do we search for biological alternatives for plant parasitic nematodes control? Because the world is calling for a more responsible lifestyle. Firstly, chemical synthesised pesticides are toxic compounds unavoidably used to control plant enemies. Conversely, these toxic compounds cause major environmental and human health problems (Oka et al. 2000; Chitwood 2002; Tsay et al. 2004; Varma & Dubey 2008; Zhao et al. 2008). Beyond that, plant parasitic nematodes are among the most widespread pests, being frequently one of the most insidious and costly, affecting economically important crops (Whitehead 1998; Bird 2003; Bent et al. 2008). According to Oka et al. (2000), global losses attributable to nematodes were estimated at 100 billion dollars annually. Hence, the need to find environmentally-friendly alternatives to chemical synthetic pesticides is evident.

In fact, research on sustainable management strategies has been increasing and it is still an important goal to reach nowadays (Chitwood 2002; Oka 2010). Pinewood Nematode (PWN), *Bursaphelenchus xylophilus*, classified as A2 quarantine organism (Nickle et al. 1981; European and Mediterranean Plant Protection Organization 2009) causes the pine wilt disease, devastating thousands of trees worldwide, causing significant economic losses (Ryss et al. 2008; Tóth 2011). In 1979 it was found in the United States of America, from where it is native (Agrios, 2005). In Europe, it was found in Portugal, in Setúbal area, in 1999 (Mota et al. 1999; EurLex 2008). The introduction of PWN into non-native areas is primarily associated with trade and the global flow of forest products (Mota et al. 1999; Mota et al. 2009). In 2008 the PWD was detected in other areas in central Portugal, particularly in the district of Coimbra (Costa et al. 2011), and in 2009 it was detected in the Madeira island, 1000 km SW of mainland Portugal, and the entire territory is now being classified as “affected area” (Ryss et al. 2008; Amador 2011; Fonseca et al. 2012; Vicente et al. 2012).

Root-knot Nematodes (RKN), *Meloidogyne* spp., are the most cosmopolitan nematode species infecting many plant species, causing significant reductions in production yield and quality (Evans et al. 1993; Agrios 2005).

Previous studies have revealed that plants contain compounds that may alter nematode behaviour and development, including attraction or repulsion from roots, hatching stimulants or inhibitors and nematotoxicants (Huang 1985; Hussey 1985; Zacheo 1993; Halbrecht 1996; Oka et al. 2000; Chitwood 2002; Adegbite & Adesiyun 2005). Volatile compounds from plants, such as essential oils, non- water- based phytochemicals, are responsible for the plant characteristic odour and they are also allelochemicals that are harmful to other organisms, inhibiting the growth of neighbouring plants and potential crop enemies. Thus, essential oils may be an eco-friendly alternative to control plant parasitic nematodes and meet the criteria of minimum risk pesticides (Isman 2000; Environmental Protection Agency 2000; Chitwood 2002; Vicente et al. 2012).

Carvone, the main compound of the essential oil of the caraway (*Carum carvi* L.) fruit, was registered by the European Commission to be used as flavourings in foodstuffs and, therefore, it has no risk to consumers' health (OJEU). It also appears on the 'Everything Added to Food in the US' (EAFUS) list, as it was classified as Generally Recognised As Safe (GRAS) or as Approved Food Additives by the United States Food and Drug Administration (FDA). Based on this knowledge, this work aimed to study the potential of carvone to control *Bursaphelenchus xylophilus* and *Meloidogyne javanica*.

2. MATERIALS AND METHODS

2.1. Nematode inoculum

Bursaphelenchus xylophilus isolate was maintained in Petri dishes (9cm x 1cm) where *Botrytis cinerea* was previously grown in an appropriate medium culture, at room temperature and in the dark. For *B. cinerea* propagation, 1 cm² of mycelium was transferred for each Petri dish with a new culture medium. When the fungus occupied 3/4 of the Petri dish it was inoculated with small pieces of culture medium containing *B. xylophilus*.

M. javanica second – stage juveniles (J2) were obtained from egg masses obtained from tomato (*Lycopersicon esculentum* cv. Rutgers) roots, 75 days after plant inoculation.

2.2. Nematicidal activity testing

Both species nematodes were firstly transferred to excavated slides with distilled water so they could be washed up before being used in experiments.

In vitro experiments were carried out in glass blocks with the different compound concentrations in controlled conditions. The various test solutions were prepared by adding the required amount of carvone (Fluka®) to distilled water with 5000 ppm of Tween 20®, in order to increase carvone solubilisation and to obtain homogeneous solutions. Each treatment consisted of 5 replicates of 20 nematodes in 1 ml of each carvone concentration. Distilled water and distilled water with 5000 ppm Tween 20® were used as controls. To define the carvone concentrations used in this work, some previously experiments were performed testing other different compound concentrations behaviour. It was found that it was not possible to test concentrations higher than 2000 ppm, as there was no solubility even adding Tween 20®: a whitish opaque solution made observations impossible. Carvone concentrations of 10, 50, 200, 1000, 1333.3 and 2000 ppm were prepared and tested on *B. xylophilus*. For *M. javanica* carvone concentrations of 10, 50, 75, 100, and 2000 ppm were tested. Pure carvone was tested for both nematode species. Mortality was recorded at 2, 18, 24, 48, 72, and 168 hours of exposure of nematodes. Nematodes were considered immobile when they recovered mobility after being repeatedly pierced with an eyelash. Nematodes were considered dead when they did not recover movement after being repeatedly pierced with an eyelash and they did not recover movement 24 hours after being transferred to another glass block with distilled water.

2.3. Statistical analysis

Data were submitted to a one-way analysis of variance (ANOVA) followed by Tukey test at $p=0.05$ level of significance (Zar 1996).

Probit analysis was also performed and LC_{50} determined for different observation time.

3. RESULTS AND DISCUSSION

In accordance with the results showed in Figures 1 and 2, carvone, the main compound of the essential oil of caraway seeds, seems to be a potential bionematicide. In fact, an evident nematocidal effect was observed on nematode species, *B. xylophilus* and *M. javanica*.

Results obtained on two controls, distilled water and distilled water with 5000 ppm Tween20®, were similar throughout the experiment, so it can be deduced that Tween 20® was innocuous in this experiment and can be used as a carvone solvent. As mortality and immobility were nil in controls, the percentage of cumulative mortality was coincident with the corrected percentage of cumulative mortality observed in the different carvone treatments. Thus, the Abbott's formula was not used.

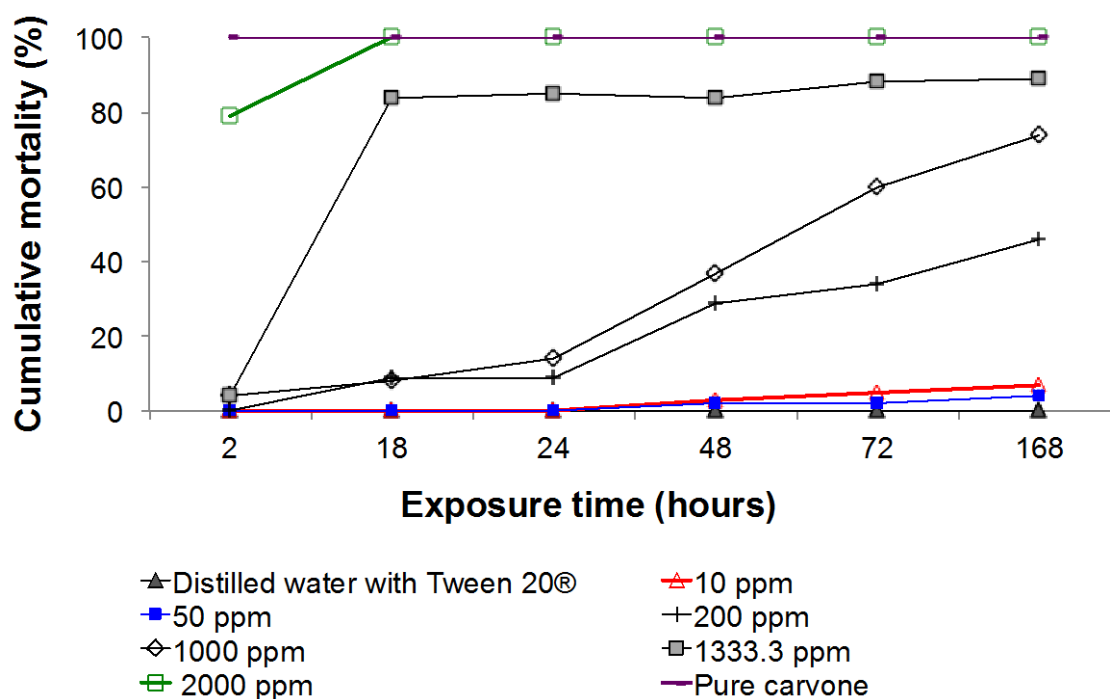


Fig 1. Percentage of cumulative mortality of *Bursaphelenchus xylophilus* exposed to different concentrations of carvone

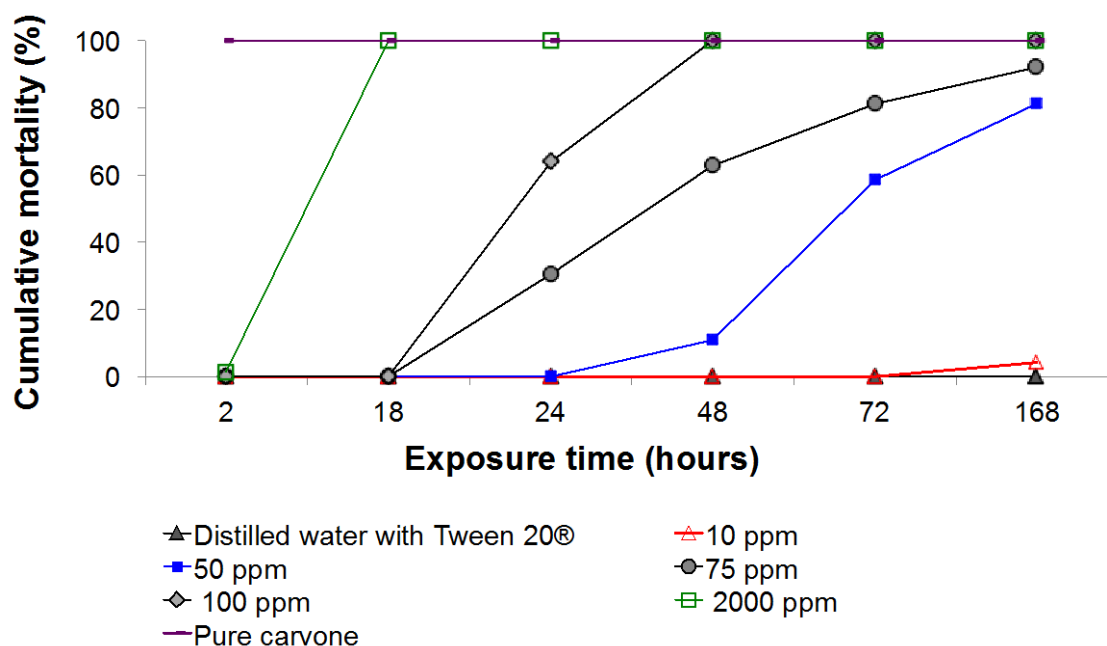


Fig 2. Percentage of cumulative mortality of *Meloidogyne javanica* J2 exposed to different concentrations of carvone

One-hundred-percent mortality was achieved on both nematode species after two hours of pure carvone exposure.

For both nematode species 100% mortality was also registered on 2000 ppm carvone concentration, at 18 hours. Although, two hours after exposure nematodes of both species were either dead or immobile: for *B. xylophilus* there was 79% mortality and 21% immobile; for *M. javanica* there was 1.3% mortality and 98.7% immobile. A nematostatic effect is also important from the nematode control point of view, since if the nematode stays immobile it cannot either eat or reproduce.

B. xylophilus in 1333.3 ppm carvone concentration exhibited 96% immobility two hours after exposure. Although, at 18h after exposure, 84% mortality was registered it increased gradually until 89%, at 168 hours. For this species, 200 and 1000 ppm concentrations caused comparable mortality values 18 hours after exposure, 9 and 8%, respectively. However, for the other observation time, the higher concentration caused higher mortality: 46 and 74%, at 168 hours. For the lowest two concentrations, 10 and 50 ppm however, 7 and 4% mortality was observed at 168h of exposure.

For *M. javanica* it was observed a dose response effect of carvone. For 50, 75 and 100 ppm at 48 hours after exposure was registered 10.7, 62.7 and 100% mortality, respectively. At 168 hours, 81.3 and 92% mortality were registered in 50 and 75 ppm, respectively. For the lowest concentration, 10 ppm, mortality was only registered at 168 hours, 4%. However, the living J2 had reduced mobility.

These results seem to indicate that RKN, *M. javanica*, was more sensitive to the active ingredient, carvone, than the PWN, *B. xylophilus*, once at 50, 75, 100 ppm concentrations a higher percentage mortality was registered for *M. javanica* over experiment time. This fact is evident on tables 1 and 2 where the Probit analysis determined LC_{50} values are showed. For all the time exposure, the LC_{50} values were always higher for *B. xylophilus* than for *M. javanica*. The results also revealed that, in a general way, the effect of carvone was dose-responsive, i.e, higher concentration caused higher mortality either on *B. xylophilus* or *M. javanica*.

There are a significant number of studies reporting nematicidal effect of plant compounds and extracts on plant parasitic nematodes (Huang 1985; Hussey 1985; Zacheo 1993; Halbrendt 1996; Oka et al. 2000; Chitwood 2002; Adegbite & Adesiyun 2005; Choi et al. 2006; Choi et al. 2007a, b, c; Kong et al

2007; Park et al. 2007; Andrés et al. 2012). In fact, new strategies for plant disease control represent a major challenge for pest science researchers in order to develop new formulations in a context of sustainable agriculture with ecological concerns.

Table 1. LC₅₀ values of carvone for *Bursaphelenchus xylophilus*

	Exposure time (hours)					
	2	18	24	48	72	168
LC 50 (ppm)	-	1180	1160	1100	750	640
LC 50 (mg/mL)	-	1.18	1.16	1.1	0.75	0.64

LC₅₀ value at two hours of exposure was not determined because there were no concentrations with mortality percentage values between the limits.

Table 2. LC₅₀ values of carvone for *Meloidogyne javanica*

	Exposure time (hours)					
	2	18	24	48	72	168
LC 50 (ppm)	-	-	89.4	67.1	47.3	29.2
LC 50 (mg/mL)	-	-	8.9E-05	6.7E-05	4.7E-05	2.9E-05

LC₅₀ values at two and 18 hours of exposure were not determined because there were no concentrations with percentage mortality values between the limits.

The present study is consistent with other evidence that revealed that constituents of plants or plant compounds have nematicide activity; among these compounds are the terpenes such as carvone, the main compound of caraway fruit (Oka et al. 2000; Chitwood 2002; Choi et al. 2007a; Abbas et al. 2009).

As a general concern, it is a must to find natural formulations that could help on plant enemies' control, which is a difficult task to accomplish, due to economic, environmental and technical issues, as well as, to restrictive legal regulations. The results obtained in this work seem sufficient to warrant further studies on the mode of action of carvone on these two economically important plant parasitic nematode species. Actually, plants are a potential source of natural based pesticides that could possibly be used as alternative to synthetic chemical pesticides.

4. CONCLUSIONS

The nematostatic and nematicidal effects of carvone on *B. xylophilus* and *M. javanica* nematodes indicate that this phytochemical may be a promising natural and environmentally-friendly alternative nematicide to conventional chemical nematicides. Nevertheless, further experiments should be conducted to evaluate the activity of this compound on hatching and plant infectivity to better understand its nematicidal potential on both nematode species, so it can become a candidate as an active ingredient for a new formulation to be used to control the Pine Wilt Disease and Root Knot Nematode, which already cause, and are still causing, significant economic losses in Portugal and throughout the world.

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