Phytopathologia Mediterranea (2017), 56, 1, 127–132
DOI: 10.14601/Phytopathol_Mediterr-19314

SHORT NOTES

In vitro nematicidal activity of naphthoquinones against the root-lesion nematode *Pratylenchus thornei*

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Summary. The root-lesion nematode *Pratylenchus thornei* is a widely distributed and important parasite of cereals and legumes. As migratory endoparasites, *P. thornei* are difficult to manage because they enter and leave host roots and may remain quiescent inside roots or in soil when conditions are unfavourable for plant growth. The number of available nematicides to manage these nematodes is restricted, so new, effective and eco-friendly sustainable management strategies are needed. The effects of naphthoquinones (juglone, 1,4-naphtoquinone and plumbagin) produced by some plants species, including walnut (Juglandaceae), were assessed against *P. thornei*. An additional treatment of a mixture of juglone and 1,4-naphtoquinone (2:1, w/w), was included because these compounds are frequently found at these propotion in walnut extracts. Juveniles and adult nematodes were exposed to different concentrations of each naphthoquinone and nematode mortality was assessed. Juglone and 1,4-naphthoquinone (at 500 ppm) were more effective than plumbagin, and gave 100% mortality after 24 h of exposure. A synergistic effect was not detected when juglone and 1,4-naphthoquinone (2:1, w/w) were combined. Estimated lethal concentrations causing 50% *P. thornei* mortality (LC_{50}s) (72 h exposure) were: 134.7 ppm for juglone, 161.2 ppm for 1,4-naphthoquinone, 207.6 ppm for juglone + 1,4-naphthoquinone (2:1, w/w), and 178.8 ppm for plumbagin. This study has demonstrated the nematicidal potential of these naphthoquinones against *P. thornei*, and has shown that walnut residues may be valuable sources for extraction of these compounds.

Key words: bionematicide, juglone, mortality, 1,4-naphthoquinone, plumbagin.

Introduction

The root-lesion nematode (RLN) *Pratylenchus thornei* Sher & Allen, 1953 is a widely distributed and common species, and an important plant-parasitic nematode (PPN) of cereals and legumes (Castillo and Vovlas, 2007). Symptoms associated with the presence of this nematode usually involve stunting, lack of vigour and nutritional deficiency symptoms in host leaves (McDonald and Nicol, 2005; Sikora *et al*., 2005). The management of RLNs is particularly challenging because, as migratory endoparasites, juveniles and adults move actively in soil and can enter and leave the roots of suitable plant hosts several times during their life cycles. Chemical control is a frequently used strategy to manage these PPNs. However, in recent years the number of nematicides available has been reduced due to environmental concerns. The increasing restrictions posed by governments on the use of synthetic nematicides have encouraged the development of alternative, effective, eco-friendly and sustainable methods to manage *Pratylenchus* spp. and other PPNs.

Plant-derived phenolic compounds are promising options for nematode management, being often

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specific to target species, biodegradable, and less toxic than conventional nematicides (Chitwood, 2002; Khalil, 2014). An important group of phenolic compounds are the naphthoquinones (juglone, 1,4-naphtoquinone and plumbagin) produced by some plants species, including trees belonging to the Juglandaceae. Extracts rich in naphthoquinones such as juglone (5-hydroxy-1,4-naphthoquinone) and 1,4-naphthoquinone can be obtained after walnut processing (Inbaraj and Chignell, 2004; Jakopič *et al*., 2007; 2009). Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) is another occurring naphthoquinone occurring in Plumbaginaceae, but also in black walnut (*Juglans nigra*) (Inbaraj and Chignell, 2004). Walnut extracts have been shown to be effective against a range of organisms, including root-knot nematodes (RKNs) (McKenry and Anwar, 2003), and the nematicidal activity of pure naphthoquinones have been demonstrated (Mahajan *et al*., 1985; Dama, 2002; Maleita *et al*., 2017). However, the effects of walnut extracts on RLNs are less clear. Tests with NatureCur®, a commercial product derived from wood of *Juglans* sp., showed that the product inhibited movement of *P. penetrans in vitro* and reduced nematode numbers on strawberry roots in a glasshouse experiment (Pinkerton and Kitner, 2006). In another study, a walnut extract failed to suppress *P. penetrans* when it was established in raspberry (Zasada *et al*., 2010). Nevertheless, in both studies the type and/or concentration of naphthoquinones in these extracts were not determined. Although the nematicidal activity of pure naphthoquinones has been already documented on RKNs, no studies have been conducted on RLN. As sedentary endoparasites, most of RKN life cycles occur inside roots, whereas the migratory RLNs are more exposed to soil environments. Since the biology of RKNs differs from RLNs, the effects of naphthoquinone compounds may be also be different and should be evaluated.

The objectives of this study were to evaluate the *in vitro* impacts of three naphthoquinones (juglone, 1,4-naphthoquinone and plumbagin), and a mixture of juglone and 1,4-naphthoquinone, on the mortality of *P. thornei* mixed stages, and to assess the potential of these compounds for nematode control. This knowledge will provide a basis for further investigations focusing the extraction of these compounds from agricultural residues, such as waste deriving from walnut processing.

Materials and methods

A *P. thornei* isolate was obtained from corn (*Zea mays* L.) roots collected in Portugal, and reared *in vitro* on Petri dishes (5 cm diam.) containing surface-sterilised carrot discs, following the protocol of Castillo *et al*. (1995) with some modifications. Fresh organic carrots were washed with sterile tap water; surface sterilized with 70% ethanol, flamed inside a laminar flow cabinet. The discs were then peeled, sliced transversely (10 mm thick) and transferred into Petri dishes (9 cm diam.). To prevent contamination, the Petri dishes containing the carrot discs were then left under UV light for 5 h. Single discs were then transferred onto 5 cm diam. dishes sealed with parafilm and kept for 5–7 d at 25ºC until nematode inoculation. Two and half months later, when nematodes were observed on the surface of carrot discs, the discs were transferred to a 20 µm sieve and washed in sterile tap water. The discs were discarded and a nematode suspension was obtained by rinsing the sieve with tap water. *Pratylenchus thornei* identification was confirmed by morphology, and molecular analysis using species-specific primers (Al-Banna *et al*., 2004; Yan *et al*., 2008).

Naphthoquinone compounds (juglone, 1,4-naphthoquinone and plumbagin; purity ≥ 95%, w/w; Sigma-Aldrich) were solubilised in Triton X-100 (Sigma–Aldrich) for 3 d at 37ºC, under agitation at 5000 ppm, to obtain final concentrations of 500, 250, or 150 ppm. Sterile tap water and Triton X-100 5,000 ppm were included as controls. Twenty RLNs, juveniles and adults, were handpicked with the aid of a dissecting microscope and placed on a glass-staining blocks containing 1 mL of each compound concentration, and then maintained in a moist chamber in the dark at room temperature. Each treatment (different naphthoquinone concentrations and controls) consisted of five replicates and the experiment was repeated three times. Nematode mortality was monitored for 3, 6, 12, 24, 48, and 72 h after exposure (hae). Nematodes not showing movement when disturbed with a bristle were transferred to sterile tap water and considered dead if they remained completely motionless in water for 24 h. Since mixtures of different naphthoquinones can be found in walnut extracts, an additional treatment of juglone + 1,4-naphtoquinone (2:1, w/w) was included, because these two compounds are usually found at this proportion in *J. regia* walnut husk extracts obtained with 40% food grade ethanol (Jakopič *et al*., 2007). The com-

pounds were mixed to obtain final concentrations of 500, 250, or 150 ppm.

Data on nematode mortality were converted to percentage cumulative mortality, corrected by Schneider Orelli's formula (equation 1) with reference to Triton X-100 5,000 ppm aqueous solution as the experimental control.

$$\text{Cumulative mortality} = \left(\frac{\text{\% mortality in treatment - \% mortality in control}}{\text{100 - \% mortality in control}}\right)\times 100 \quad (1)$$

The effects of the different naphthoquinones and concentrations on nematode mortality were compared in one-way analyses of variance (ANOVA) followed by post-hoc Fisher LSD statistical tests. Statistical analyses of the data were performed using Statsoft Statistica version 7 for Windows. Data derived from the 24 and 72 h observations were subjected to Probit analysis (Finney, 1971) using PriProbit v.1.63 software, and the lethal concentrations causing 50% mortality (LC_{50}s) were calculated.

Results and discussion

The mortality of *P. thornei* in Triton X-100 5,000 ppm, the surfactant used for solubilisation of compounds, was not significantly different from that observed in the sterile tap water control. However, *P. thornei* mortality was significantly affected by the three naphthoquinones at all concentrations ($P<0.05$). In general, a response to increases in concentration resulted in increased *P. thornei* mortality, for all tested compounds, except for juglone at 250 and 150 ppm, and 1,4-naphthoquinone at 250 ppm ($P>0.05$; Figure 1). Furthermore, 100% nematode mortality was achieved in 1,4-naphthoquinone at 500 ppm, within 24 h of exposure (Figure 1). To the other treatments, at 500 ppm, values of mortality of approx. 90% were obtained. These results demonstrated that the three naphthoquinones reduced nematode survival, in short periods following exposure.

Since naphthoquinones, such as juglone, can be degraded in soil by bacteria (Schmidt, 1988), it is important that RLN control can occur before compound degradation and nematode root penetration. In addition, rapid degradation of these compounds may be advantageous to minimise impacts on other soil microbial communities. A strategy based on the application of naphthoquinone compounds/extracts at pre-planting, may be effective for reducing soil populations of *P. thornei*. A similar strategy was used by Potter *et al*. (1998) to reduce the numbers of *P. neglectus* in soil, by pre-planting soil incorporation of *Brassica* tissues, rich in glucosinolates.

In the present study, the combination of juglone + 1,4-naphthoquinone (2:1, w/w), at 500 and 250 ppm, was more effective for reducing *P. thornei* survival than exposure to juglone alone. The mixture also had less effect than 1,4-naphthoquinone alone, suggesting that the nematicidal activity of 1,4-naphthoquinone may have been reduced by the presence of juglone. Although a synergistic effect was not observed using the mixture of juglone + 1,4-naphthoquinone (2:1, w/w) that is found in walnut extracts (Jakopič *et al*., 2009), it is possible that different ratio mixtures of these two compounds may produce different results. The concentration of phenolic compounds in walnut extracts can be variable due to seasonal, genetic and ecological factors (Solar *et al*., 2005; Cosmulescu and Trandafir, 2011). Therefore, the development of a formulated product based on pure naphthoquinone compounds, extracted from walnut residues from farming and processing activities, or naphthoquinone-enriched extracts, could be preferable to the use of walnut extracts.

Estimated LC_{50}s for the compounds, at 24 h, ranged from 200.0 ppm (1,4-naphthoquinone) to 467.7 ppm (juglone + 1,4-naphthoquinone). At 72 hae, the LC_{50}s was 134.7 (juglone) and 207.6 ppm (juglone + 1,4-naphthoquinone) (Table 1). Thus, juglone and 1,4-naphthoquinone were the most effective of

Table 1. Estimated values of lethal concentrations (ppm) necessary to give 50% mortality (LC_{50}) of *Pratylenchus thornei* mixed stages at 24 or 72 h after exposure to juglone (J), 1,4-naphthoquinone (N) or plumbagin (P).

Time after exposure (h)	Compound			
	J	N	J:N[a]	P
24	317.0	200.0	467.7	399.8
72	134.7	161.2	207.6	178.8

[a] Juglone + 1,4-naphthoquinone (2:1 w:w), according to Jakopič *et al*. (2007).

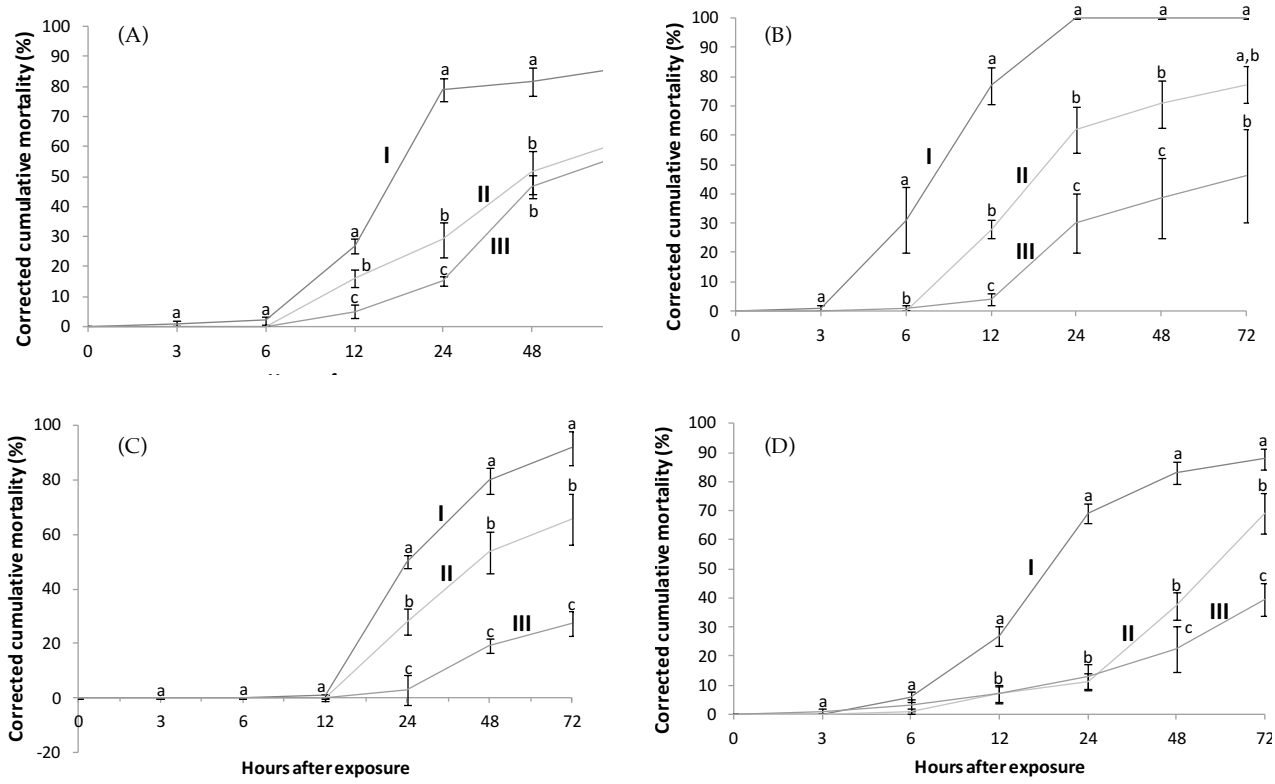

Figure 1. Mean corrected cumulative mortality (%) of *Pratylenchus thornei* mixed stages exposed to different concentrations of juglone (A) and 1,4-naphthoquinone, alone (B) or combination (2:1 w:w) (C), and plumbagin (D). Treatment concentrations are: I, 500 ppm; II, 250 ppm; or III, 150 ppm. Data were corrected with reference to Triton X-100 5,000 ppm, and are means of five replicates. Vertical bars indicate standard errors. Means for each compound accompanied by the same letter do not differ (P>0.05) according to the Fisher LSD or Kruskal-Wallis tests.

the tested compounds to induce *P. thornei* mortality (Table 1). These results agree with those obtained recently with the RKN *Meloidogyne hispanica* (Maleita *et al*., 2017). In general, *M. hispanica* was more susceptible to naphthoquinone compounds than *P. thornei*. *Meloidogyne hispanica* was killed after 3 h of exposure, whereas for *P. thornei* this effect took longer. However, different results were obtained by Dama (2002) when testing the effects of juglone, plumbagin and lawsome (2-hydroxy-1,4-naphthoquinone) on the *in vitro* mortality of *M. javanica*. At 24 hae, plumbagin was more effective than juglone, causing 100% mortality in *M. javanica* second-stage juveniles. It is possible that nematode susceptibility to naphthoquinone compounds is variable among different species of PPNs.

During the assays reported here, reductions in nematode activity were observed after the first hours of exposure to naphthoquinones. Live nematodes remained still and only recovered movement when touched with a bristle or transferred to tap water, whereas dead nematodes showed straight or a slightly bent shapes. Changes in the shape of dead nematodes have been observed in *P. goodeyi* exposed to acetone and water extracts of *Solanum nigrum* and *S. sysimbriifolium* (Pestana *et al*., 2014). Effects on mobility and morphological changes have been attributed to the presence of compounds such as alkaloids with central nervous system effects and saponins which alter membrane permeability (Pestana *et al*., 2014).

Previous research with *Caenorhabditis elegans* showed that plumbagin has activity as an oxidative stressor, and has been associated with a repression in nematode growth, development and reproductive processes, promoting energy conservation and repair instead of energy expenditure (Hunt *et al*., 2011). Depending on certain conditions (for example, pH

and compound concentration), naphthoquinones such as juglone and plumbagin are known to exhibit broad-range toxic effects on living organisms and have different biological activities, such as anticancer, antibacterial, antifungal, antiviral, anti-inflammatory and antipyretic properties (Strugstad and Despotovski, 2012). However, their potential activities may directly result from their specific chemical structures. Despite their chemical similarities, the naphthoquinones tested in the present research have different molecular structures. 1,4-naphthoquinone lacks R5-OH and R2-CH3 groups, juglone lacks the R2-CH3 group, and plumbagin possesses R5-OH and R2-CH3 groups. These configurations may lead to distinct redox and equilibrium properties for these compounds. Further studies are required to clarify the modes of action of different naphthoquinone compounds against PPNs.

Although the nematicidal activity of the three phenolic compounds and whole walnut extracts have been documented for RKN (Dama, 2002; Maleita *et al*., 2017), no studies on their effects on RLN have been reported. The *in vitro* tests conducted in the present study demonstrated that juglone, 1,4-naphthoquinone and plumbagin have nematicidal activity against *P. thornei*. These compounds were able to reduce *P. thornei* survival after short exposure periods. Since the lifecycle of this nematode occurs both in soil and inside roots, the application of naphthoquinones-based compounds/extracts at pre-planting may be effective to reduce population densities of *P. thornei* and *Meloidogyne* spp. in soil. Esteves *et al*., (2015) observed RLN species, such as *P. penetrans*, *P. neglectus*, *P. thornei* and *P. crenatus*, in potato root samples, coexisting with RKNs. Future studies should investigate the nematicidal effects of these compounds when applied into soil, possible effects on plant growth in naphthoquinone-sensitive plants, assessment of the impacts on other soil communities, as well as the potential use of walnut residues for extraction of bioactive/nematicide compounds.

Acknowledgments

This research was financed by FCT/MEC through Portuguese national funds and co-funding by the FEDER, within the PT2020 Partnership Agreement and COMPETE 2020 (projects UID/BIA/04004/2013, FCOMP-01-0124-FEDER-027960, PEst-C/EQB/UI0102/2013), and by IATV-Instituto do Ambiente, Tecnologia e Vida. I. Esteves (SFRH/BPD/68856/2010), C. Maleita (SFRH/BPD/85736/2012), L. Fonseca (SFRH/BPD/101325/2014) and M. Braga (SFRH/BPD/101048/2014) are funded by post-through doctoral fellowships from MEC National funding and The European Social Fund through Programa Operacional Capital Humano.

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Accepted for publication: March 9, 2017
Published online: May 10, 2017