EFFECTS OF NEEM, *Azadirachta indica*, SEEDS' FILTRATE ON HATCHING, MORTALITY, AND INFECTIVITY OF ROOT-KNOT NEMATODE, *Meloidogyne javanica*

PENGARUH FILTRAT BIJI NIMBA, Azadirachta indica, *TERHADAP TETASNYA TELUR, MORTALITAS, DAN INFEKTIFITAS NEMATODA PURU AKAR*, Meloidogyne javanica

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ABSTRACT

Effect of neem (*Azadirachta indica*) seeds' filtrate on hatching, mortality, and infectivity of *Meloidogyne javanica* was investigated. Six concentrations of filtrate (1, 4, 10, 25, 50, and 100 g/L) were evaluated on hatching of *M. javanica* for 3, 7 and 14 days. Mortality of *M. javanica* was studied by exposing J2 in four concentrations of filtrate (10, 25, 50, and 100 g/L) for 24 hours. Then infectivity of nematode was investigated after incubated J2 in 25-g/L filtrate for 1, 6, 12, and 24 hours. All experiments were completely randomized designed and, where appropriate, data were analyzed with analysis of variance followed by honestly significant different test at 5% significant level. Research results show that filtrate inhibited egg hatch with inhibition gradually increased with time and was concentration-dependent. Exposure to 1 g/L filtrate caused least inhibition of J2 hatching, and 50- and 100- g/L filtrate produced 100% inhibition at all times. Second-stage juvenile mortality was concentration-dependent and 100% mortality was achieved by exposure to 100-g/L, 50-g/L, and 25- g/L filtrates within 9, 22, and 24 hours, consecutively. Infectivity of J2 decreased by 50, 73, and 84% after nematodes had been exposed to 25 g/L filtrate for 6, 12, and 24 hours, respectively.

Key words: Neem, *Azadirachta indica,Meloidogyne javanica,* root-knot nematode, and toxicity.

ABSTRAK

*Pengaruh filtrat biji nimba (*Azadirachta indica*) terhadap tetasnya telur, mortalitas, dan infektifitas* Meloidogyne javanica *telah diteliti. Filtrat dengan enam konsentrasi (1, 4, 10, 25, 50, dan 100 g/L) diuji pengaruhnya pada tetasnya telur* M. javanica *selama 3, 7, dan 14 hari. Mortalitas nematoda diteliti dengan memaparkan J2 pada empat konsentrasi filtrat (10, 25, 50, dan 100 g/L) selama 24 jam. Infektifitas nematoda diuji setelah J2diinkubasi dalam filtrat 25-g/L selama 1, 6, 12, dan 24 jam. Semua percobaan dirancang secara acak lengkap dan, dimana perlu, data dianalisa dengan analisis keragaman dilanjutkan dengan uji beda nyata jujur pada taraf nyata 5%. Hasil penelitian menunjukkan bahwa filtrat menghambat penetasan telur dengan penghambatan yang meningkat seiring waktu dan konsentrasi. Pemaparan pada filtrat 1-g/L menyebabkan hambatan tetas yang paling sedikit, dan filtrat 50- dan 100-g/L menghambat penetasan 100% pada setiap pengamatan. Mortalitas J2 tergantung pada konsentrasi filtrat dan mortalitas 100% dicapai dengan filtrat 100-g/L, 50-g/L, and 25- g/L masing-masing setelah pemaparan selama 9, 22, dan 24 jam. Infektifitas J2 berkurang 50, 73, and 84% setelah nematoda dipaparkan pada filtrat 25-g/L masing-masing selama 6, 12, dan 24 jam.*

Kata kunci: Nimba, Azadirachta indica, Meloidogyne javanica, *nematoda puru akar, dan toksisitas*

INTRODUCTION

Plant-parasitic nematodes are considered as major pests and the cause of serious yield losses on a wide range of crops in many countries, particularly in the tropics and subtropics (Luc *et al*., 1990). It is estimated that 12.3% of the 50% of potential crop losses caused by pests to be caused by nematodes and more of this damage is in the developing than developed countries (Sasser and Freckman, 1986). Among the plant parasitic nematodes, root knot nematodes *Meloidogyne* spp., are economically the most important, limiting agricultural productivity and quality (Sasser and Carter, 1985). This nematode can cause 24–38% loss on a tomato crop where sequential cropping of susceptible crops is practiced with up to four crops per year. In the absence of effective control root-

knot nematodes can cause total crop failure (Sikora and Fernandez, 1990).

Historically, management of nematodes has been done by using plant resistance, crop rotation, cultural practices, or chemical nematicides (Chitwood, 2002). Most chemicals used as nematicides are toxic and/or volatile, with poor target specificity and less-than-perfect human or environmental safety. Also, there are serious concerns over issues such as ground water contamination and atmospheric ozone depletion (Thomas, 1996). These problems associated with the use of nematicides have promoted the screening of plants for antihelminthic activity.

Neem (*Azadirachta indica*), belonging to Meliaceae family, leads the list of plants with the highest potential for this purpose (Kumar *et al*., 1995). Neem contains several biologically active chemicals called limonoids such as azadirachtin, nimbin, salannin, azadirachtol, nimbidin, gedunin, and others. These compounds are responsible for diverse activities such as insect antifeedant, insect growth disrupting, insecticidal, nematicidal, fungicidal, and bactericidal (Schmutterer 1990). Azadirachtin, is the most potent and the most abundant (0.2–0.6% (w/w)) chemical found in seed kernels of neem. The bitter extracts from neem kernels obtained by alcoholic extraction contain various bioactive limonoids and are widely used in biopesticidal formulations (Govindachari *et al*., 1990).

Neem seed kernel extract suppresses the feeding, growth and reproduction of insects (Schmutterer, 1990). Butterworth and Morgan (1971) first isolated the tetranortriterpenoid azadirachtin from *A. indica* seeds, which primarily showed antifeedant activity and later, regulatory effects on larval development and metamorphosis. Due to their relative selectivity, neem products can be recommended for many integrated pest management (IPM) programs (Schmutterer, 1990; Mordue and Blackwell, 1993).

Studies in recent years have revealed insecticidal effects of many different limonoids. The specific effects studied include growth inhibition, feeding inhibition, molt inhibition and insect growth regulation. Most studies have focused on the insect orders Coleoptera, Diptera, Heteroptera, Lepidoptera and Orthoptera. Azadirachtin or azadirachtin containing extracts have been shown to affect over 400 species of insects and mites (Schmutterer, 1990; Mordue and Blackwell, 1993). Azadirachtin possessing antifeeding and repellent or masking action against harmful insect were studied enough earlier (Mordue and Blackwell, 1993), but their efficacy against nematodes, particularly root-knot nematodes, *Meloidogyne* spp. has not been tested.

The objectives of the present study were to assess the effects of a hydrophilic water seed filtrate of neem on hatching, mortality, and infectivity of *M. javanica*.

MATERIALS AND METHODS

Extraction of neem seeds: The neem seeds were collected in Gunung Sari, Mataram, Lombok. The seeds were dried at ambient temperature and stored in the dark, at room temperature. They were then ground to a fine powder using a porcelain mill. One hundred grams of this powder (corresponding to ca. 750 g wet weight) was suspended in 1 L of distilled water in a 2-L flask for 96 hours in the dark. The suspension was filtered with Whatman No. 1 filter paper. The filtrate was stored at 5^{o}C until use.

Nematode population: The *M. javanica* population, obtained from naturally infected tomato (*Lycopercon esculentum*) plants from Pejeruk, Ampenan, Lombok, was maintained and increased on a susceptible host, *L. esculentum*, Tomato cv. Mutiara.

Effect of filtrate on hatching of M. javanica eggs: The filtrate of neem seeds was diluted with distilled water to obtain final concentrations of 1, 4, 10, 25, 50, and 100 g/L. Egg masses were picked from roots infected tomato with *M. javanica*, transferred to distilled water, and torn with a needle to release the eggs, from which those embryonated with second stage juvenile (J2) were pipetted into Petri dishes with thin layer of water agar (2%). The water in the surface of agar that came with eggs was dried by exposing the open dishes in a laminar air flow. Each treatment consisted of four replicates of 50 eggs of which then were wetted by 1 ml of each filtrate concentration. The dishes were kept in a most plastic container, in the dark, at room temperature. Hatched eggs were counted after 3, 7, and 14 days in treatments and controls (distilled water).

Data on hatching were converted to percentage cumulative hatching inhibition which were counted as a/b x 100% (a = percentage hatching inhibition in filtrate – percentage hatching inhibition in control, and b = 100 – percentage hatching inhibition in control) (Abbott, 1925). Data were further analyzed by analysis of variance,

followed by Honestly Significant Different test at 5% significant level.

Effect of filtrate on mortality of M. javanica: Filtrates of 10, 25, 50, and 100 g/L were prepared as above. Second-stage juveniles were obtained from egg masses picked from infected tomato roots by placing them in a 300-µm-pore nylon sieve in a Petri dish (5 cm) containing distilled water at room temperature. Twenty-four hours later, the resulting J2 suspension was discarded and those that hatched in the subsequent 24 hours were used for experiment. They were hand-picked into 100 µl of distilled water on glass slides using a bristle and checked for normal movement using stereomicroscope. Twenty-five J2 per treatment were then transferred to the surface of water agar (2%) with thin layer of each filtrate concentration; distilled water served as control. Each treatment was replicated four times. This experiment was conducted in the dark, in a moist plastic container, at room temperature (28^{o}C). Observations were made at 1, 3, 6, 9, 12, 18, 20, 22, and 24 hours. Second-stage juveniles that did not move when touched with a bristle were transferred to distilled water. They were considered dead if they still failed to react to probing with a bristle one hour later. Mortality data were converted to percentage cumulative mortality.

Effect of filtrate on infectivity of M. javanica: The infectivity experiment was conducted on tomato plants grown in 200-cm^3 plastic pots filled with a mixture of autoclaved sand: autoclaved soil (1:1). One hundred recently hatched J2 were handpicked into 500 µl distilled water on a glass slide using a bristle. Then, 500 µl filtrate of 50-g/L was added to obtain a 25-g/L concentration. The J2 were incubated in a moist plastic container in the dark at room temperature for 1, 6, 12, or 24 hours. Second-stage juveniles exposed in this way were transferred to distilled water immediately before they were inoculated near the roots of a tomato plants with two pairs of true leaves. Thus, plants were never in contact with the extract. Each exposure treatment consisted of five replicates, as did the unexposed control.

The pots were placed randomly assigned positions in an open place with temperatures ranging from 26 to 33^{o}C for 7 days. They were watered daily and fertilized once (one day before nematode inoculation) with complete water soluble fertilizer (N:P:K 15:15:15). The plants were then uprooted, and their roots were washed free of soil and stained with acid fuchsin. Nematodes in the entire root system of each plant were counted using a stereomicroscope, and infectivity was expressed as a percentage of the number that infected roots of control plants.

RESULTS AND DISCUSSION

Results

Effect of filtrate on hatching: All six filtrate concentrations inhibited egg hatching at each exposure period (Table 1); the average hatching of control nematodes was 92.8 ± 6.6%. Generally, inhibition of hatching gradually increased with time and was concentration-dependent. Exposure to 1 g/L filtrate caused least inhibition of egg hatching, and 50- and 100- g/L extract produced 100% inhibition at all times.

Table 1. Cumulative percentage hatching inhibition of *Meloidogyne javanica* eggs exposed to different periods and filtrate oncentrations of neem (*Azadirachta indica*) seeds.

Filtrate concentration (g/L)	Cumulative hatching inhibition (%) after different exposure periods (days)		
	3	7	15
1	16.7a*)	21.3a	25.2a
4	64.3b	74.8b	76.5b
10	86.9c	89.7c	92.2c
25	90.6d	94.6d	95.3d
50	100.0e	100.0e	100.0e
100	100.0e	100.0e	100.0e
$HSD_{0.05}$	8.93	5.11	4.62

*) Numbers followed by the same letters at the same column are not significantly different.

Effect of filtrate on mortality: One-hundred-percent mortality was achieved by exposure to the three filtrate solutions, although it occurred after different times of exposure. The 100- g/L filtrate induced 100% mortality within a 9-hour exposure, whereas the 50- g/L filtrate elicited the same effect in 22 hours and the 25- g/L filtrate in 24 hours (Figure. 1). Second-stage juvenile mortality was concentration dependent.

Effect of filtrate on infectivity: The 25-g/L filtrate inhibited the infectivity of exposed *M. javanica* J2 on a susceptible host (Figure. 2), with infectivity decreased by 15% after exposure periods as short as 1 hour. A 6-hour exposure of J2 to this extract concentration decreased the number of nematodes inside roots by 50%, and a 70% decrease occurred with a 12-hour exposure period. The

greatest observed effect on infectivity was an 84% decrease after a 24-hour exposure period.

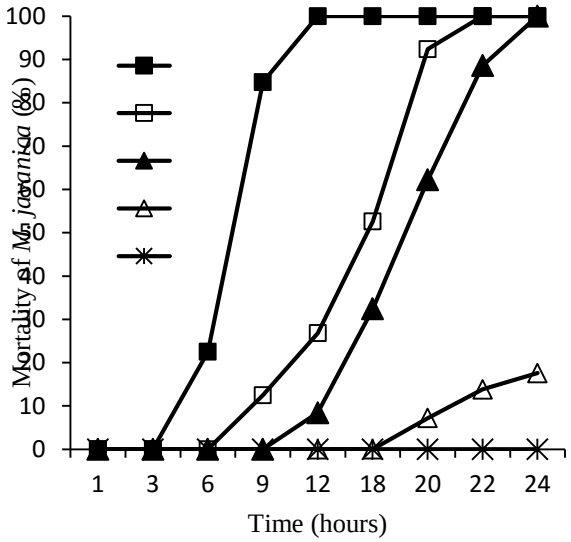

Figure 1. Percentage cumulative mortality of *Meloidogyne javanica* J2 exposed to different concentrations of filtrate of neem (*Azadirachta indica*) seeds. A) 100-g/L filtrate. B) 50-mg/L filtrate. C) 25-g/L filtrate. D) 10-g/L filtrate. E) distilled water (control).

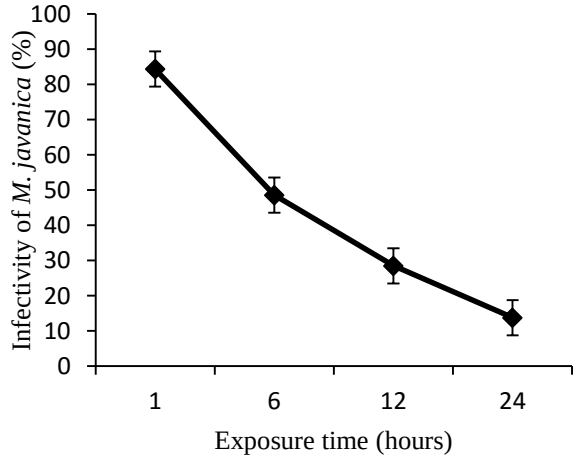

Figure 2. Infectivity of *Meloidogyne javanica* on tomato cv. Mutiara after exposure to filtrate of neem (*Azadirachta indica*) seeds at 25 g/L. Bars are the values of $HSD_{0.05}$.

Discussion

Information on the effects of neem extract or filtrate on root-knot nematodes, *Meloidogyne* spp., in detail is very limited. There is a conflicting evidence about the toxicity of pure substances extracted from neem. Khan *et al*. (1974) and Devakumar *et al*. (1985) claimed that direct toxicity of neem formulations to nematodes is attributed to chemicals like azadirachtin. On the other hand, Grandison (1992) could not observe any direct effects of the purified substances from neem seed like azadirachtin, salanine and nimbin on J2 of *M. javanica*. The results of the present study are similar to the findings of Khan *et al*. (1974) and Devakumar *et al*. (1985) but not that of Grandison (1992).

The 100% hatching inhibition induced by the 50- g/L preparation of the filtrate in this study is as high as any reported for filtrate of other plant species, *Arthemesia* species. Water extracts of fresh shoots of *Arthemesia annua* and of *A. pallens* inhibited hatching of *M. incognita* by approximately 99.6% and 99%, respectively, at an extract concentration of 333 g/L (Pandey, 1990). This is about six times more concentrated than the filtrate used in this study. A water extract of dried shoot of *A. absinthium* induced 98.7% hatching inhibition of *M. incognita* at 50 g/L (Korayem *et al*., 1993), a concentration similar to the data presented here. A dichloromethane extract (0.1 g/L) of *Xanthosoma sagitifolium* produced 85% hatching inhibition of *M. megadora* after 28 days exposure (Gulhano *et al*., 1997). Corrected hatching inhibition (Abbott, 1925) may vary along exposure time, reflecting not only the extract activity but also the influence of hatching in control.

Since hatching of nematode eggs depends on the activity of J2 inside eggs, any substance that able to inhibit eggs to hatch must be able to penetrate the egg shell and influence J2. Therefore, azadirachtin as the most potent and the most abundant (0.2–0.6% (w/w) chemical found in seed of neem (Isman, 2006) and other biologically active chemicals that can inhibit egg hatching must be able to penetrate nematode egg shell and influence J2. Although its mechanism is not clear yet, it is suggested that azadirachtin has a toxic effect on J2 resulting in inability of J2 to break egg shell and then egg does not hatch.

The toxic effect of azadirachtin becomes pronounced when the effect of filtrate on J2 mortality was taken into account. All the three filtrate concentrations tested caused 100% mortality although it occurred after different times of exposure (Figure. 1). Reduction of exposure time as the increase of filtrate concentrations indicates that J2 mortality was concentration dependent.

The nematicidal effect of the filtrate was concentration-dependent. As the mortality bioassay

was an acute toxicity test and it is assumed that no extract degradation occurred during the 24 hours of the test, nematotoxicity is attributed to the filtrate constituents. The mortality of nematode J2 within 24 hours is very important to be taken into account since any type of extract or filtrate would be very difficult to maintain in the field longer than 24 hours. For this reason, the effect on infectivity was tested only for 25-g/L filtrate concentration.

Result of this study shows that a 50% decrease in infectivity after 6 hours of exposure of *M. javanica* J2 to 25 g/L water filtrate of *A. indica*. Few studies have assessed the effects of plant extracts or compounds on plant infection by nematodes. A 4-hour exposure of *M incognita* J2 to a 20-ppm solution of thiarubrine C, isolated from roots of *Rudbeckia hirta*, resulted in no infectivity of tomato *Lycopersicon esculentum* cv. Rutgers (De Viala *et al*., 1998). However, a true comparison of activity will be possible only after isolation and purification of active compounds from the *A. indica* filtrate, as water filtrate of *A. indica* a very complaex mixture of compounds (Schmutterer, 1990). The observed gradual decrease in infectivity with increasing exposure time is probably due to *M. javanica* J2 becoming less mobile or vigorous after exposure to nematotoxic compounds in the filtrate. The 25-g/L filtrate solution caused mortality only after a 12-hour exposure (Figure. 1), so the decreased in infectivity within that exposure time may have been due to a J2 behavioral change at a concentration below the lethal range. There is a possibility that lethal effects would have been observed eventually if J2 were transferred to water after short exposure periods. Behavioral changes at sub-lethal concentrations are usually an indicator of neurotoxicity (Williams and Dusenbery, 1990). Infectivity testing could be a useful tool in determining the mode of action of active compounds in the filtrate. Also, the protocol of exposing the J2 to the test filtrate in solution, followed by a test of infectivity, may serve as a laboratory first approximation of what would occur after a drench treatment; active filtrate or compounds could then be subjected to pot experiment.

The present results also seem sufficient to warrant studies on purification and the mode of action of bioactive compounds of *A. indica*, and on the efficacy of other extracts on this and other species of plant-parasitic nematodes. Bioassay-guided isolation of active compounds along with complementary tests such as microplot experiments will help us clarify the means of exploiting *A. indica* nematotoxicity in order to develop inexpensive, natural, and environmentally friendly nematicides.

CONCLUSION

Neem seeds' filtrate inhibited egg hatch with inhibition gradually increased with time and was concentration-dependent. Exposure to 50- and 100- g/L filtrate produced 100% inhibition at all times. Second-stage juvenile mortality was concentration-dependent and 100% mortality was achieved by exposure to 100-g/L, 50-g/L, and 25-g/L filtrates within 9, 22, and 24 hours, consecutively. Infectivity of J2 decreased by 50, 73, and 84% after nematodes had been exposed to 25 g/L filtrate for 6, 12, and 24 hours, respectively.

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