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Effective control of neem (*Azadirachta indica A. Juss*) cake to plant parasitic nematodes and fungi in black pepper diseases *in vitro*

Tác động của bánh dầu neem (Azadirachta indica A. Juss) lên tuyến trùng và nấm bệnh ký sinh cây hồ tiêu ở điều kiện in vitro

Research article

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Neem cake is a product of the cold pressing from the neem kernels to obtain neem oil. Bio-active substances from neem cake extracted solutions were evaluated for their potential to control the root knot nematodes and other pests of plants. In this study different concentrations of the solution extracted from neem cake was tested against the second stage juveniles of the plant parasitic nematode *Meloidogyne* spp. and four phytopathogenic fungi: *Rhizoctonia solani, Sclerotium rolfsii, Collectotrichum spp.* and *Phytopthora capsici*. Toxicity of neem cake extractions is represented by the EC_{50} value for the second-stage juvenile (J2) of *Meloidogyne* spp. and the four phytopathogenic fungi via Probit analysis. A 5% dilution of the solvent extracting from neem cake already caused 100% larval mortality after 24 hours exposure. Undiluted neem cake extraction effectively inhibited the growth of the four phytopathogenic fungi. The EC_{50} value of neem cake on J2-larvae of *Meloidogyne* nematode and on the fungi *Rhizoctonia solani, Sclerotium rolfsii, Collectotrichum spp. and Phytophthora capsici* was 0.51, 0.74, 0.30, 0.51 and 4.33%, respectively.

Bánh dầu neem là sản phẩm của quá trình ép nhân hạt neem để lấy dầu. Các hoạt chất sinh học từ dịch chiết bánh dầu neem đã được đánh giá có tiềm năng lớn trong phòng trừ tuyến trùng nốt sưng và các loài dịch hại khác của nhiều loại cây trồng. Trong nghiên cứu này các nồng độ dịch chiết khác nhau của bánh dầu neem đã được thử nghiệm khả năng diệt tuyến trùng (ấu trùng tuổi 2 thuộc giống Meloidogyne spp.) và ức chế 4 loài nấm bệnh như: Rhizoctonia solani, Sclerotium rolfsii, Collectotrichum spp. và Phytopthora capsici. Độc tính của dịch chiết bánh dầu neem được biểu diễn bởi giá trị EC_{50} đối với ấu trùng tuổi 2 của tuyến trùng Meloidogyne spp. và các loài nấm bệnh thông qua phân tích Probit. Dịch chiết bánh dầu neem ở nồng độ 5% đã làm chết 100% cá thể IJ2 của Meloidogyne spp sau 24 giờ phơi nhiễm. Dịch nguyên chất bánh dầu neem ức chế cả 4 loài nấm bệnh. Giá trị EC_{50} của bánh dầu neem lên ấu trùng tuổi 2 của Meloidogyne spp và các loài nấm bệnh Rhizoctonia solani, Sclerotium rolfsii, Collectotrichum spp. and Phytophthora capsici tương ứng là 0.51, 0.74, 0.30, 0.51 và 4.33%.

Keywords: neem cake, biocontrol, root knot nematode, anti-fungal activity, plant extracts, phytopathogenic fungi, *Meloidogyne* spp.

1. Introduction

Plant parasitic nematodes cause great economic losses to agricultural crops worldwide. Among them root-knot nematode, especially the genus *Meloidogyne* are most harmfull in the tropics to many plants, such as black pepper trees. In the past only chemicals were usedas nematicides. However, the abuse of chemical pesticides has

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negative impact on the beneficial insects as well as on human health (Vu Dang Khanh, 2003). Therefore, to conserve and establish an agricultural system with ecological sustainability, it is necessary to develop biological methods to not only control the nematode populations, but also to ensure productivity, quality of plants and to reduce environmental pollution.

Neem cake is the residual part of the process to compress the neem seeds to obtain oil and it is considered as a source of useful organic fertilizer. This kind of fertilizer not only provides nutrition for plants but also controls the root-knot nematodes. Moreover, neem cake can be used as inhibitors to control many species of pathogenic fungi (Singh *et al.*, 1984; Sartaj *et al*., 1995; Vu Trieu Man and Le Luong Te, 1998; Farooq *et al*., 2008).

Currently neem cake and many other derivates are used for controlling plant parasitic nematode (Musabyimana T., and R.C. Saxena, 1999; Nazir *et al.*, 2007). Many publications showed that the biological activity of neem cake has the ability to reduce the populations of root-knot nematodes, such as *Meloidogyne incognita* in the soya plant and *Meloidogyne javanica* in both tomato and and soya trees. The powder of neem seeds are also used to control *Meloidogyne arenaria* and *Pratylenchus penetrans* in tomato plants. Some authors also experimented on neem cake mixed with other materials such as wheat straw, sawdust and NPK fertilizer to control root-not nematode effectively (Ramarao P., Usharaja, 1983; Randhawa N.S, B.S. Parmar, 1996).

Hence this study aims to evaluate the effect of neem cake for controlling root-not nematode in Vietnam and its antagonistic potential against phytopathogenic fungi.

2. Materials and methods

2.1 Materials

2.1.1 Preparation of neem cake extracted solutions

Fresh seeds were collected from ripe fruits harvested from 4 years old neem (*Azadirachta indica* A. Juss) trees growing in Ninh Thuan province, Vietnam then cleaned and dried in the shade to 10-15% moisture content. The dried neem seeds were transferred to the Experimental Laboratory of the Institute Tropical Biological, where the seeds were separated from the pellets. Neem cake and neem oil were obtained simultaneously by using cold-pressing vegetable oil machine (KOMET Model D 85 – 1G, Germany) from compressing neem seed kernel. A part of the neem oil was used to produce bio-pesticides, and the rest part was used as material for this study.

100 grams of neem cake was grinded and soaked into 200 ml alcohol 96^0 for 4 hours and then extracted by filtering the solution 4 times through the sterilized millipore 0.45µm filter, the rest was discarded. All filtrations were mixed up together and vacuum extracted by rotation of the obtaining solution 100 ml was taken as neem enrichment extract (NEE) for testing. This extracted solution was used undiluted and diluted with pure sterilized water to the following concentrations: 2.5, 5, 10 and 20% (Vu Van Do, 2007).

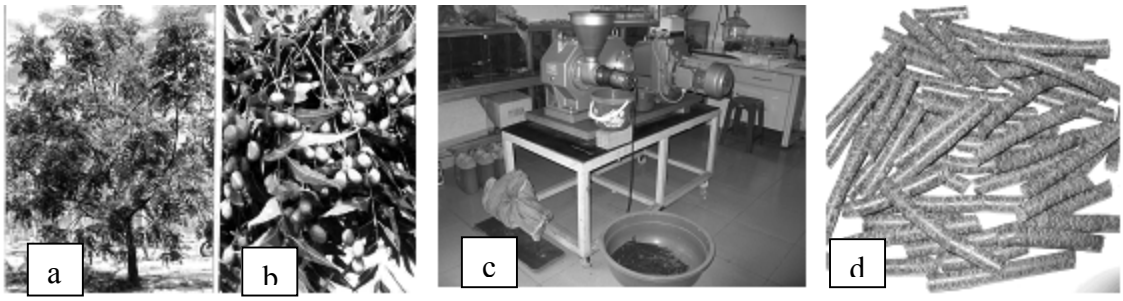


Figure 1. a) Neem tree planted in Ninh Thuan, Vietnam; b) neem fruits; c) cold-pressing machine; d) neem cake obtained by cold-pressing via Komet (Germany)

2.1.2 Extraction of juveniles

The second stage juveniles of *Meloidogyne* spp. were extracted from the roots of black pepper plants in Thuan Hanh village (Dak Song district, Dak Nong province) by modified Baermann tray extraction method using sieves with 0,5mm mesh size put into petri dish (diameter 90mm, 15mm high) with soft papers on it. Clean roots of black pepper were put on the sieve and water was added to the level of submerging the roots. The petri dish was covered and put into the incubator room during 48 hours at 28^0C. The living nematodes penetrated through the sieve and settled down at the bottom of the petri dish (Nguyen Ngoc Chau, 2003; Coyne et al, 2007).

2.1.3 Pathogenic fungi

Four species of phytopathogenic fungi: as *Rhizoctonia solani, Sclerotium rolfsii, Collectotrichum spp.* and *Phytopthora capsici* were received from Department of Plant Protection (Ho Chi Minh Agricultural and Forest University) and was maintained in PGA until needed.

2.2 Methods

2.2.1 Root knot nematode bioassay

Ten millilitre of the undiluted and the following concentrations: 2.5, 5, 10 and 20% of neem cake extract were separately poured into petri dishes and 1 ml of suspensions containing about 20 J2 individuals were added to each petri dish. All treatments were replicated three times. The petri dishes were incubated at room temperature. Percent mortality was calculated after 6, 24 and 48 hours. All the data collected were analyzed using analysis of variance and means separated with the Least Significant Difference Test in a MSTATC software.

2.2.2 Fungi bioassay

PGA (Potato Glucose Agar) medium was used for maintaining the cultures on sterile petri dishes. The undiluted and each dilution of neem cake extracted solutions were passed through a membrane filter (0.45 μm dimension) to avoid any bacterial and fungal contamination, then 1ml of the test extraction solution is transferred to the sterilized dish and 20 ml of the molten PGA medium (45^0C) is added, and this mixture combining solution in the PGA medium is kept at cool temperature. PGA medium without neem cake extraction solution is used as the control. A uniform fungal mycelium is obtained by introducing a 5-mm-diameter stainless-steel cork borer into the center of the petri dish. Each treatment is replicated three times. The plates were incubated at room temperature for 4 days. After the incubation period, two perpendicular diameters of the fungi were linearly measured in mm and the mean diameter was calculated. The percentage of growth inhibition was calculated as:

$$\text{The percentage of growing inhibition} = \frac{Dc - Dt}{Dc} x100$$

In which:

D_c: the average colony diameter of fungi in the control experiment.

D_t: the average colony diameter of fungi in the treatment experiment.

2.2.3 Data process

The mortality rate of *Meloidogyne* spp. second stage juveniles was determined after every hour as well as the percentage of growing inhibition of the fungi. The EC_{50} for nematodes and the IC_{50} for fungi was calculated by the method of Probit analysis, the data were processed via the Microsoft Excel (Nguyen Ngoc Kieng, 1992).

3. Results and discussion

3.1 The effect of neem cake on the *Meloidogyne* spp. second stage juveniles

Table 1 shows that most of the *Meloidogyne* spp. second stage juveniles already died after 6 hours when using 5% or more of the neem cake extracted sollutions and was 100% at 10% or more of the extracted sollution. The mortality of *Meloidogyne* spp. second stage juveniles is 87% and 100% after 6 hours and 24 hours exposed in 5% concentration, respectively. At 2.5% concentration, the mortality of *Meloidogyne* spp. second stage juveniles was nearly 80% after 48 hours. These results indicate that neem cake extracted solution is very effective to control the root-not nematodes. In fact, neem cake containing the biological substance (azadirachtin, salannin, nimbin) and its derivative compound belonging to the group of triterpenoid, limonoid, etc. have been proven to contain strong toxicity for plant parasitic nematode (Keshav *et al.*, 1996; Nazir Javed, 2008; Adegbite A.A., and S.O.Adesiyan, 2005) but do not harm the beneficial microorganism in the soil (Muhammad Abid, 1996).

Table 1. Percentage of J2 mortality in testing with neem cake extracted solution

Concentration of neem cake extracted solution (%)	The second stage juveniles mortality (%) after		
	6 hours	**24 hours**	**48 hours**
0 (control)	0,00 ± 0,00d	0,00 ± 0,00c	0,00 ± 0,00c
2,5	24,17 ± 0,76c	63,0 ± 0,87b	77,83 ± 2,57b
5	87,5 ± 1,26b	100 ± 0,00a	100 ± 0,00a
10	100 ± 0,00a	100 ± 0,00a	100 ± 0,00a
20	100 ± 0,00a	100 ± 0,00a	100 ± 0,00a
100	100 ± 0,00a	100 ± 0,00a	100 ± 0,00a
CV (%)	0,83	0,31	0,00
LSD	1,227	0,523	-

The percentage was transformed to arcsin $(x)^{1/2}$ before using for statistical process. Other characters after data column show the significant difference with $p \leq 0.01$ by ranking LSD.

Compared to Adegbite *et al.* (2005), our results are much better: larval mortality already occurs at 5% concentration after 6 hours, whereas in the experiment of Adegbite *et a*l (2005) larval mortality only is observed at 100% concentration or undiluted neem cake extractions. The other level of concentration like 20%, 10% and 5% leads to reduction of the larval mortality percentage from 90% to 1.7%. In the meantime, neem cake extracts from Ninh Thuan, Vietnam kill almost of second-stage juveniles at the level of concentration 10% -100%, even at 5%, in the time of 24 hours or 48 hours. At the level lower than 5% (2.5%), it also controls 2nd Juvenile effectively with the percentage of mortality from 24,17-77,83% in 6, 24 and 48 hours.

This means that neem cake extracted solution from neem (*Azadirachta indica* A. Juss) tree originating from Ninh

Thuan, Vietnam can be very useful as bio-control method against *Meloidogyne* spp. second stage juveniles.

3.2 The anti-fungal activity of neem cake extracted solutions on pathogenic fungi

Data in table 2 show that the phytopathogenic fungus *Rhizoctonia solani* was already inhibited by 17% dilution of NEE: Water (1:30), and original undiluted extracted solution inhibits completely *R. solani* (100%).

Table 2. The radial growth diameter (mm) and inhibition percentage of fungi (%): *R. solani, S. rolfsii, Collectotrichum spp.* and *Phytophthora capsici* by neem cake extracted solutions

		The percentage of neem cake extracted solution (%)				
		Control	NEE	NEE:water (1:10)	NEE:water (1:20)	NEE:water (1:30)
Rhizoctonia solani	Radial growth diameter (mm)	90	0	55	70	75
	Inhibition percentage (%)	0	100	39	22	17
Sclerotium rolfsii	Radial growth diameter (mm)	90	0	28	30	58
	Inhibition percentage (%)	0	100	69	67	36
Collectotrichum spp	Radial growth diameter (mm)	40	2	15	22	27
	Inhibition percentage (%)	0	95	62.5	45	32.5
Phytophthora capsici	Radial growth diameter (mm)	55	18	40	60	60
	Inhibition percentage (%)	0	67	27	0	0

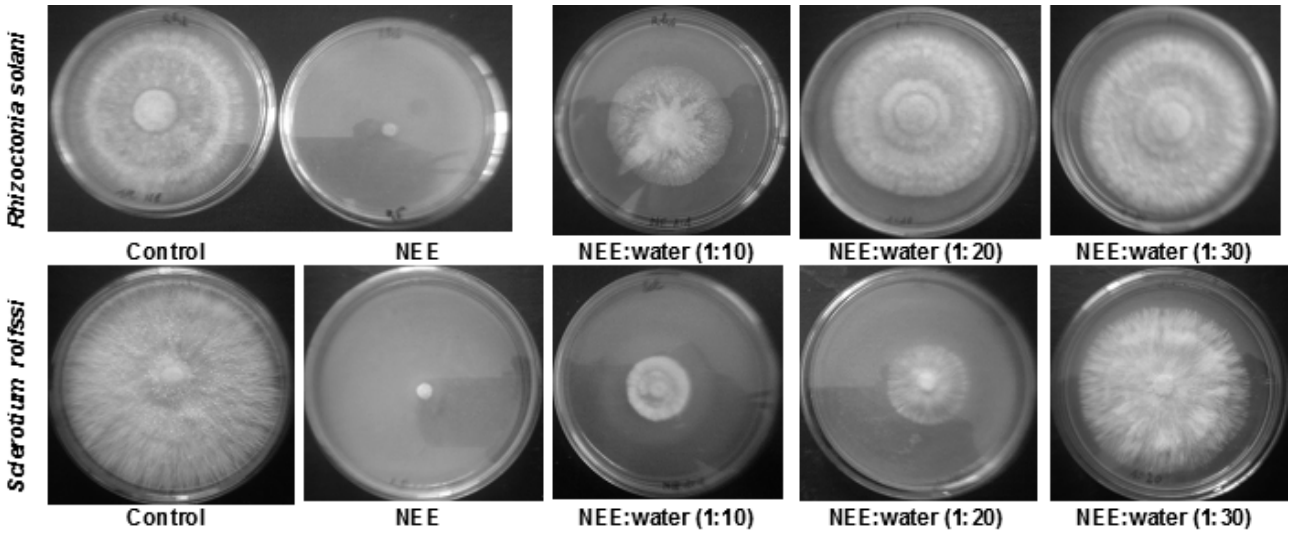
Notes: NEE – neem enrichment extract; NEE:water – dilution of neem cake extracted solution with ratio 1:10, 1:20 and 1:30.

For *Sclerotium rolfsii*, has a characteristic growth by white fibers spreading out as rays around and it covers almost the petri dish surface only after 4 days (Figure 2). The inhibiting effect of dilution extracted solution NEE:water at 1:30 and 1:10 concentrations was respectively 36% and 69%. The ability of 100% inhibition of the NEE solutions was also found. According to Ramarao (1983) besides neem cake, extracted solution from castor-oil plant and groundnut cake are also good to use for controlling the fungus *S. rolfsii* on the wheat plants.

With regard to the phytopathogenic fungus, *Collectotrichum spp.* grows by forming suckers in the host and the speed of development is rather low (Figure 2). The corresponding inhibiting effects are 32%, 45% and 62% with a dilution rate of NEE:water being 1:30, 1:20 and 1:10 respectively. Original extracted solution (NEE) can inhibit this type of fungus up to 95%.

Figure 2 shows that *Phytopthora capsici* grows and creates patches of cobweb or star colonies, growing quite slowly compared to other types of disease fungi. However, the inhibiting effect of undiluted neem cake is only 67% and the inhibiting effect diminishes at lower concentrations becoming 27% at the dilution of NEE: water being 1:10 and further dilutions have not any inhibiting effect. This result corresponds to the findings of Jeyarajan *et al*. (1987), besides *Phytopthora capsici*, they also evaluate the difference of additional compost, sawdust and neem cake on land against *Fusarium solani* causing root rotten disease in the green bean plants. Singh and Vyas (1984) also studied the influence of some kinds of pressure cakes as mustard plant, castor-oil plant, neem and mahua to *Phytophthora parasitica* and *P. piperina*. The results showed that almost all of the pressure cake inhibits growth of those phytopathogenic fungi.

Study results show that neem cake inhibits the growth of *Sclerotium rolfsii* most effectively, followed by *Rhizoctonia solani* and *Collectotrichum spp*. The growth rate of phytopathogenic fungus *Phytopthora capsici* is only slightly inhibited by neem cake.



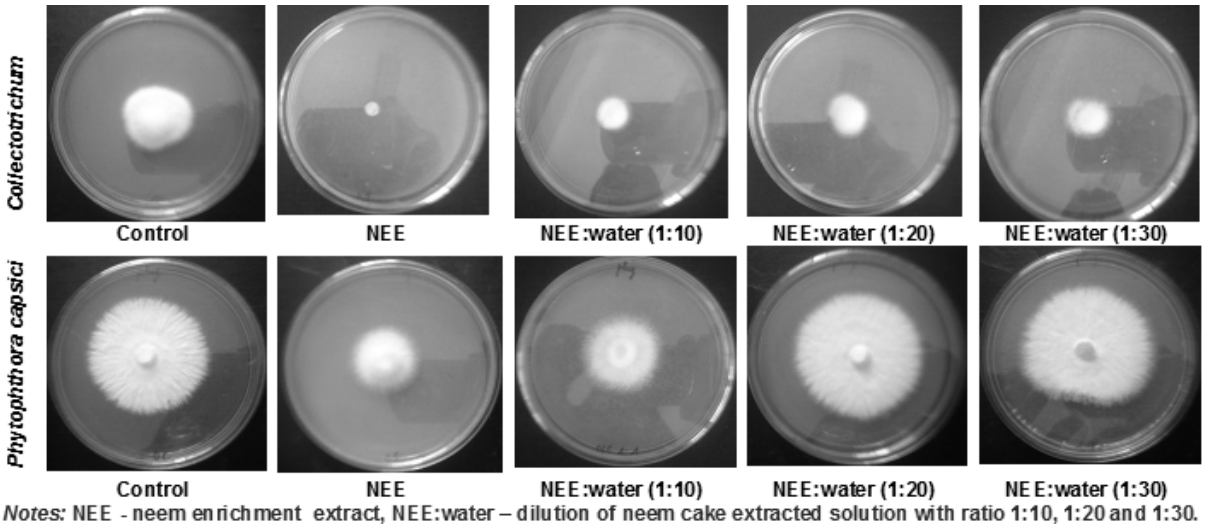


Figure 2. The effect of different concentrations of neem cake extracted solution on the growth of *Rhizoctonia solani*, *Sclerotium rolfsii*, *Collectotrichum spp.* and *Phytophthora capsici*

3.3 The value EC_{50} (50% Effect Concentration) of neem cake

The EC_{50} value is an important parameter to evaluate the toxicity level of neem cake extracted solution on second stage juveniles of the root-knot nematode *Meloidogyne* and the phytopathogenic fungi *Rhizoctonia solani, Sclerotium rolfsii, Collectotrichum spp.* and *Phytophthora capsici*.

EC_{50} values are presented in Table 3.

Table 3. Value EC_{50} of neem cake extracted solution on second stage juveniles and pathogen fungi

		Statistic function (Y = a + bX)	*Correlation coefficient (R)*	*Value EC_{50} (gram/100 ml)*
Meloidogyne spp. second stage juveniles	*After 6 hours*	Y = -6.92338 + 6.253031 X	*0,99*	*0,81*
	After 24 hours	Y = -11.2162 + 9.207056 X	*1,0*	*0,58*
	After 48 hours	Y = -8.73705 + 8.024782 X	*1,0*	*0,51*
Phytopathogenic fungi	*R. solani*	Y = 0.943564 + 2.171388 X	*0,90*	*0,74*
	S. rolfsii	Y = 2.333924 + 1.797774 X	*0,86*	*0,30*
	Collectotrichum spp	Y = 2.953501 + 1.19974 X	*0,89*	*0,51*
	P. capsici	Y = -1.50745 + 2.468208 X	*0,95*	*4,33*

The results in Table 3 show that the EC_{50} value for *Meloidogyne* spp. juveniles decreases with increasing exposure time value prooving that the effectivity of neem cake increases with time.

The IC_{50} value shows that neem cake extracted solution inhibits the 4 fungi *Rhizoctonia solani, Sclerotium rolfsii, Collectotrichum spp.* and *Phytophthora capsici*. The neem cake extracted solution inhibits most strongly the growth of *Sclerotium rolfsii* (0,30%) and is less effective towards the growth of *Phytophthora capsici* (4,33%).

4. Conclusion

This research demonstrated that Ninh Thuan neem cake extracted solution has high ability to control the *Meloidogyne* spp. second stage juveniles and can inhibit the growth of 4 phytopathogenic fungi in black pepper (*Piper nigrum* L.). The results also show that these neem cakes contain bioactive substrates. Those substrates are considered as main factors to control nematodes and fungi in the black pepper plants.

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