Fungitoxic Effect of Neem Extracts on Growth and Sclerotial Survival of *Macrophomina phaseolina in vitro*

R.C. Dubey[1], Harish Kumar[1], R.R. Pandey[2]

[1.] Department of Botany and Microbiology, Gurukul Kangri University, Haridwar- 249 404, Uttarakhand, India
[2.] Department of Life Sciences, Manipur University, Canchipur, Imphal -795 003, India
profrcdubey@gmail.com, pandey.rr@rediffmail.com

Abstract: In this study, we examined the *in vitro* effect of extracts of different neem (*Azardirachta indica* A. Juss) plant parts such as leaf, bark, oil cake and neem oil on the growth, mycelial yield and sclerotial survival of *Macrophomina phaseolina* (Tassi) Goid. causing charcoal rot of soyabean (*Glycine max* L.) by poisoned food technique. The radial growth of *M. phaseolina* was promoted by the autoclaved neem extracts. Maximum enhancement in growth of the fungus was observed at 10 % concentration of cake extract followed by leaf and bark extracts. However, the neem extracts sterilized through sintered glass filter (G-5) showed inhibitory effect on growth and mycelial biomass yield of *M. phaseolina*. Neem oil was found to be most toxic followed by cake, leaf and bark extracts. Effect of neem extract on sclerotial survival of *M. phaseolina* was found inhibitory after 2 and 4 days of incubation. Sclerotia treated with neem oil did not germinate, hence resulted in 100% inhibition. Volatile and non-volatile fungistatic effects of different neem extracts (5 %) were also inhibited sclerotial germination of the test fungi. Maximum inhibition in sclerotial germination was observed with cake extract after 96 h of incubation followed by leaf and bark extract. The effect of volatile fungistasis was more pronounced than non-volatile fungistasis. Volatile fraction of cake extracts caused 97 % inhibition in sclerotia germination after 96 h but non-volatile effect of cake extract after 48 h was negligible. [Journal of American Science 2009; 5(5):17-24]. (ISSN: 1545-1003).

Keywords: Neem extracts; *Macrophomina phaseolina;* mycelial growth; sclerotial germination

1. Introduction

Neem (*Azadirachta indica* A. Juss.) tree also known as "Indian Lilac", belongs to the family Meliaceae, has attracted special interest of scientists due to the presence of a variety of bioactive compounds (Tewari, 1992). It has a great potential to control various phytopathogenic fungi and, therefore, has much prospect to be used as a good fungicide. Several neem based commercial products are also available in market. The use of neem cake and neem leaves as a soil treatment measure have produced good results against various soil borne fungi like *Pythium aphanidermatum* and *Rhizoctonia solani* (Khan et al., 1974), *Fusarium oxysporum* (Kannaiyan and Prasad, 1981), *Calletotrichum atramentarium* (Singh, 1986). Effectiveness of neem extracts and oil as a fungicide has earlier been reported by several workers (Ilyas et al., 1997; Sharma and Basandrai, 1997; Lokhande et al., 1998). Dubey and Kumar (2003) found almost similar effect of azadirachtin, mancozeb and bavistin on *in vitro* growth and sclerotial survival of *M. phaseolina*.

M. phaseolina (Tassi) Goid. is an important phytopathogen distributed worldwide and causes charcoal rot on more than 500 plant species. Moreover, this fungus survives in soil by multicellular jet black microsclerotia produced enormously during parasitic phase and/or saprophytic phase (Dubey and Upadhyay, 2001). Sclerotia of *M. phaseolina* are such a potent resting body that each and every cell is potential to germinate and cause disease.

The aim of the present study was to investigate the effect of different neem extract and volatile and non-volatile fractions on germination and survival of *M. phaseolina* sclerotia.

2. Material and Methods

2.1. Isolation of fungus

M. phaseolina was isolated from diseased roots of soyabean (*Glycine max* L.) collected from Imphal and Nainital districts of India. Roots were cut into 1 cm long pieces and were dipped in 0.1 % $HgCl_2$ solution for 1 min followed by serial washing with sterilized distilled water for 10 times. The pieces were

dried by using sterile filter paper and then placed onto Potato Dextrose Agar (PDA) medium containing Petri dishes under aseptic condition. The plates were incubated for 5-6 days at 30±1 ^{o}C. The pathogen was maintained on PDA slants for further use.

2.2. Preparation of neem extracts

Fresh and healthy leaves were collected from neem tree, washed with distilled water and dried in shade. The leaves were used to make a paste with distilled water (1:1, w/v) by using mixer/grinder (Model Maharaj, Whiteline). Bark was gently removed from the tree, dried in shade and powdered in mixer/grinder. Powder was mixed with distilled water in a ratio of 2:1 (w/v) and left overnight to allow the constituents to get dissolved in water. Bark extract was sterilized by sintered glass filter (G-5). Oil cake was collected from an Expeller mill, powdered and mixed with distilled water (1:1, w/v). Thereafter, mixture was squeezed out and extract was collected in sterile glass vials. The extract was sterilized as above. Extract and oil were kept at 4 ^{o}C and were used within two days.

2.3. Effect of neem extracts on growth of *M. phaseolina*

Effect of extracts on growth of *M. phaseolina* was studied by poisoned food technique based on radial growth and mycelial yield.

2.3.1. Colony diameter

Czapek Dox agar plates were prepared and 1, 5 and 10% concentration of each extract (leaf, bark and cake) was added separately. The concentration of neem oil was 0.1, 0.5, 1.0 %. Each plate was inoculated with an agar block (5 mm diam) of *M. phaseolina* growing on PDA. The plates were incubated at 30±1 ^{o}C for 4 days. Growth inhibition (%) was measured by the formula: 100 × (C-T)/C, where C = growth in control, T = growth in treatment.

2.3.2. Estimation of mycelial biomass

Czapek Dox broth was prepared for estimation of mycelial yield and 1, 5, and 10 % concentration of each extract (leaf, bark and cake) was mixed separately *in vitro*. The concentration of neem oil was the same as described above. The flasks were inoculated with 5 agar blocks (each of 5 mm diam) containing mycelial growth of *M. phaseolina*. The flasks were incubated at 30±1 ^{o}C for 10 days. Thereafter, cultures were filtered through pre-weighed Whatman filter paper No. 1. Mycelial yield was determined after drying the mycelial mat at 85 ^{o}C for 24 h. Percent loss/gain in mycelial dry weight was calculated by using the formula : 100 × (C-T)/C, where C = mycelial dry weight in control, T= mycelial dry weight in treatment.

2.4. Effect of extracts on sclerotia survival of *M. phaseolina*

2.4.1. Sclerotia production

The sclerotia of *M. phaseolina* were harvested by cellophane disc method (Ayanru and Green, 1974). PDA medium was poured into sterile Petri dishes. The cellophane paper discs were cut according to the size of Petri dishes and boiled for 30 min to remove plasticizers. The cellophane discs were gently spread onto the surface of PDA plates, inoculated with an agar block of *M. phaseolina* and incubated at 30±1 ^{o}C for 5 days. On the sixth day the cellophane paper was gently removed from the medium and sclerotia were harvested and placed onto sterile filter paper to dry. Dried sclerotia were mashed with mortar and pestle and filtered by a sieve of pour size 150 µm.

2.4.2. Survival of sclerotia

Sclerotia survival study was performed following the standard tube dilution method of Baily and Scott (1974) modified by Dwivedi and Dubey (1986). A small amount of sclerotia prepared as above was transferred into 50 ml sterile distilled water. Five ml of sclerotia suspension was transferred into a sterilized glass tube and centrifuged at 1000 ***g*** for 3 min. Supernatant was decanted and 5 ml of each extract was added separately in glass tubes in triplicate. Control set contained only 10 ml distilled water. After 2 and 4 days of incubation at 30±1 ^{o}C the sclerotial suspension (in extract) was first decanted and then washed for 4 to 6 times with sterile distilled water to remove traces of the extract from the sclerotial surfaces. Finally 5 ml distilled water was added to prepare sclerotial suspension. One ml of suspension was poured onto 2 % water agar medium, properly spread over and finally incubated at 30±1 ^{o}C for 24 h. Total number of sclerotia showing germination in each treatment was counted.

2.5. Fungistatic effect of volatile and non-volatile fractions

Sclerotial suspension was used for the volatile and non-volatile fungistasis

2.5.1. Volatile fungistasis

Volatile fungistasis was studied by inverted petriplate method (Dwivedi and Dubey, 1986). Fifteen ml aliquot of autoclaved water agar (2 %) medium was poured into sterile Petri dishes. In another set, Petri dishes containing 20 g of soil amended with different neem extract (5 %) separately were prepared and sterile distilled water was added to the soil to maintain 20 % water holding capacity. The Petri plates containing soil were kept as such for 4 days to allow the microbial activity and decomposition of neem extracts. Sclerotial suspension (1 ml) was poured onto the surface of Petri dishes containing sterile water agar medium, gently spread over and left for a few minutes to settle down the sclerotia. Excess water was decanted and evaporated and the lid of Petri plate containing 20 g of amended soil was replaced with the bottom halves of plate containing sclerotial suspension. The two bottom halves were sealed together with an adhesive tape and incubated at 30±1 ^{o}C in dark. Control set had the similar pairs devoid of soil. Germination of sclerotia was observed under microscope after 24, 48, 72 and 96 h of incubation. Inhibition of sclerotial germination was calculated statistically.

2.5.2. Non-volatile fungistasis

It was studied by agar disc method (Jackson, 1958). Sclerotial suspension was prepared as above. More water was added in amended soil kept in Petri dishes to bring it in sticky condition. Small pieces of filter paper (2x2 cm) were put onto surface of soil kept in plates. An agar block (1x1 cm) containing fungal sclerotia was kept over each filter disc. All the plates were incubated at 30±1 ^{o}C and sclerotial germination was counted after 24, 48, 72 and 96 h of incubation as described earlier.

3. Results

3.1. Effect of neem extracts on growth of *M. phaseolina*

Radial growth of *M. phaseolina* was promoted by all autoclaved neem extracts. Maximum enhancement in radial growth (42.3 %) was observed at all concentration of cake extract followed by leaf extract (33.1 %) and bark extract (29.2 %) (Table 1). Whereas, neem extract filtered through bacteria proof sintered glass filter (G-5) showed a marked inhibition in growth of the fungus. At 10 % concentration growth of *M. phaseolina* was completely inhibited by cake extract followed by leaf extract (70.6 %) and bark extract (52.6 %). Neem oil was found to be most toxic which caused 85.4 % inhibition at 10 % concentration followed by 95.9 % inhibition in radial growth at 5 % concentration of cake extract (Table 1).

Similarly, neem extracts also inhibited mycelial yield of *M. phaseolina* as compared with control. Maximum inhibition in mycelial biomass yield was observed with cake extract (97.8 %) at 10 % concentration. Oil at 1 % concentration was the most effective causing 92.6 % inhibition in mycelial yield followed by cake extract (71.5 %), while bark extract had the minimum inhibitory effect at the same concentration (Table 2).

Table 1. Effect of different neem extracts (autoclaved and unautoclaved) on growth of *Macrophomina phaseolina in vitro.*

Treatment	Growth inhibition/ enhancement* (%)					
	1 %		5 %		10 %	
	Autoclaved	Unautoclaved	Autoclaved	Unautoclaved	Autoclaved	Unautoclaved
Bark extract	12.3 ± 0.34	26.3 ± 0.27	18.6 ± 0.06	44.7 ± 0.14	29.2 ± 0.03	52.6 ± 0.06
Leaf extract	15.8 ± 0.20	36.6 ± 0.24	26.8 ± 0.14	45.3 ± 0.06	33.1 ± 0.06	70.6 ± 0.55
Cake extract	19.7 ± 0.12	79.5 ± 0.08	30.6 ± 0.02	95.9 ± 0.06	42.3 ± 0.08	100 ± 0.01
Oil*	-	34.6 ± 0.37	-	67.3 ± 0.08	-	85.4 ± 0.12

Values are mean of three replicates ± standard error. * Concentrations of oil used were 0.1 %, 0.5 % and 1.0 %

Table 2. Effect of different neem extracts on mycelial yield of *Macrophomina phaseolina in vitro*.

Treatment	Loss in mycelial dry weight (%)		
	1 %	5 %	10 %
Bark extract	25.1 ± 0.51	35.8 ± 0.01	41.9 ± 0.36
Leaf extract	52.5 ± 0.04	81.2 ± 0.02	92.0 ± 0.02
Cake extract	71.5 ± 1.01	91.1 ± 0.03	97.8 ± 0.98
Oil*	37.8 ± 0.12	63.6 ± 0.06	92.6 ± 0.02

Values are mean of three replicates ± standard error, * Concentrations of oil used were 0.1 %, 0.5 % and 1.0 %

3.2. Effect of neem extracts on survival of *M. phaseolina* sclerotia

Survival of *M. phaseolina* sclerotia was studied after 2 and 4 days of incubation. Only 23 % sclerotia survived upto 4 days when treated with cake extract as compared to control where 96 % sclerotia germination was recorded. Bark extract had least effect on survival of sclerotia permitting only 66 % germination after 4 days of incubation. Very few sclerotia showed good germination (i.e. produced >7 germ tubes/sclerotium in case of cake extract treatment). However, most of them showed poor germination showing 1-3 germ tube/sclerotium or no germination. Sclerotia treated with oil did not germinate, hence it resulted in 100 % inhibition in sclerotia germination (Table 3).

Table 3. Effect of neem extracts on survival of *Macrophomina phaseolina in vitro.*

Treatment	Germination of sclerotia (%)	
	2 days	4 days
Control	90 ± 2	96 ± 4
Bark extract	78 ± 5	66 ± 2
Leaf extract	70 ± 3	54 ± 5
Cake extract	50 ± 5	23 ± 2
Oil	NG*	NG*

Values are mean of three replicates ± standard error, *NG denotes no germination of sclerotia

3.3. Volatile fungistasis of neem extracts against *M. phaseolina*

Fungistatic effect of neem extracts (5 %) on *M. phaseolina* is shown in Figure 1A. Sclerotia germination of test fungi gradually decreased with increase in incubation time. Maximum inhibition in sclerotial germination (97 %) was observed in cake extract after 96 h of incubation. After 24 h of incubation though a little inhibition in sclerotial germination occurred as compared to control, yet most of the sclerotia showed very good germination (>7 hyphae/sclerotium) in each extract. Oil cake extract caused slightly higher decline than the other extracts (Figure 1A). After 48 h of incubation 38, 64 and 77 % sclerotia germinated in case of oil cake, leaf and bark extract, respectively in comparison to control (84 %). Highest germination rate was observed in sclerotia treated with bark extract and also in control set, whereas lower germination rate (4-7 hyphae/sclerotium) was shown by 31 and 15 % sclerotia treated with leaf and cake extracts, respectively. Upon increasing the incubation time most of the sclerotia showed poor germination (1-3 hypahe/sclerotium). Cake extract caused 86 % inhibition in sclerotia germination after 72 h of incubation. Volatile fungistatic effect of neem extracts was more potentiated after 96 h of incubation. Ninety seven percent inhibition in sclerotial germination by cake extract was recorded after 96 h of incubation (Figure 1A).

A similar result was also noticed with neem oil where sclerotial germination gradually decreased with increase in concentration of oil and incubation period (Figure 1B). Maximum inhibition (39 %) was noticed with 1.0 % oil after 96 h of incubation. The number of hyphae/sclerotium was higher after 24 h incubation which gradually decreased with increase in oil concentration and incubation period. Most of the sclerotia showed poor or no germination (1-3 hyphae/sclerotium) after 96 h of incubation.

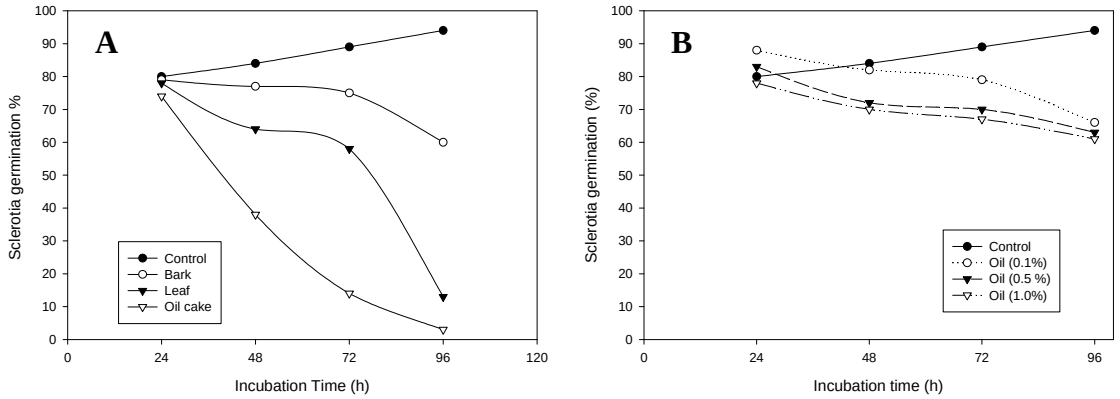

Figure 1. Volatile fungistatic effect of different (A) neem extracts (5% w/w) and (B) neem oil (0.1, 0.5, 1.0 %) on *Macrophomina phaseolina* sclerotia after 24, 48, 72, 96 h of incubation at 30±1°C.

3.4. Non-volatile fungistasis caused by neem extracts

After 24 h of incubation most of the sclerotia showed very good germination producing >7 hyphae/sclerotium (Figure 2A). Oil cake extract posed maximum inhibition on sclerotia germination (77%) after 48 h incubation followed by leaf extract and bark extract that caused 73 % and 66 % inhibition in sclerotia germination. Most inhibitory effect was found at 1.0 % concentration of neem oil which caused 32 % and 41 % inhibition after 24 and 48 h of incubation, respectively. Most of the germinated sclerotia showed poor germination (1-3 hyphae/sclerotium) or good germination (4-7 hyphae/sclerotium) after 48 h of incubation (Figure 2B).

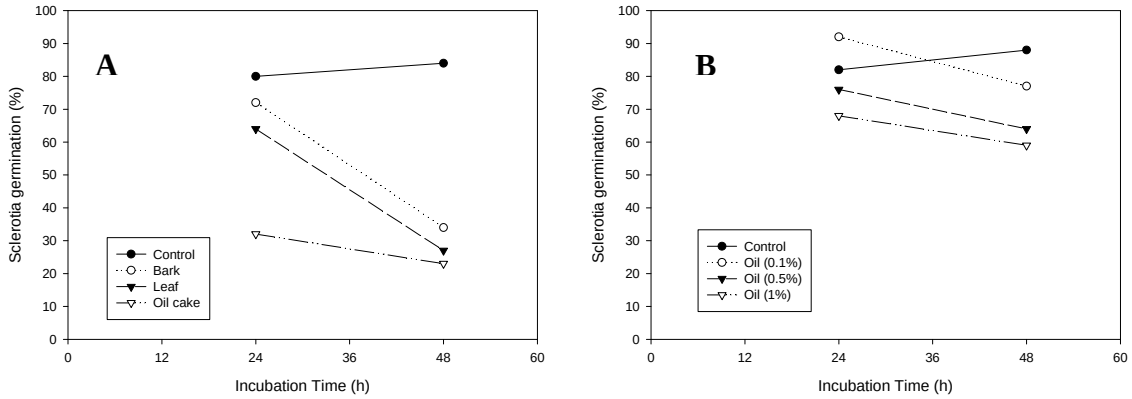

Figure 2. Non-volatile fungistatic effect of different (A) neem extracts (5 % w/w) and (B) neem oil (0.1, 0.5, 1.0 %) on *Macrophomina phaseolina* sclerotia after 24, 48 h of incubation at 30±1°C.

The non-volatile effect could not be studied after 72 h of incubation because counting of germinated sclerotia became difficult due to over growth of fast growing fungi from soil that made difficult to distinguish between hyphae of *M. phaseolina* and other fungi. By comparing it is clear that non-volatile effect on sclerotial germination was greater than volatile effect. Cake extract (5 % w/w) caused 77 % inhibition in case of non-volatile fungistasis, whereas same concentration of cake caused 62 % inhibition in case of volatile fungistasis after 48 h incubation.

4. Discussion

Neem contains a variety of chemical constituents such as nimolicinol, isolimolicinolide, azadirachtin, azadirachtol, nimlinone, nimbocinol, nimbocinone, nimocin, etc. (Tewari, 1992). Dubey and Kumar (2003) reported the fungicidal effect of azadirachtin as good as the fungicides bavistin and mancozeb. These chemicals present in plant cause deleterious effect on the microorganisms. Radial growth of *M. phaseolina* was promoted by autoclaved neem extracts. The inhibitory chemicals present in neem extract would have been denatured at high temperature due to autoclaving, which in turn promoted the growth instead of inhibition because the pathogen used the denatured chemicals as substrates. Maximum enhancement in radial growth was measured at 10% concentration of cake extract followed by leaf and bark extract. A similar result had been reported by Rao et al. (1996) who found mycelial growth promotion of *Verticillium leccani* treated with autoclaved neem extract. Neem extract filtered through bacterial proof sintered glass filter showed inhibitory effect on radial growth as well as biomass yield of *M. phaseolina*. Neem oil was most inhibitory followed by cake extract. Several bioactive compounds have been reported from different parts of neem (Tewari, 1992). The most active principle present in neem oil is azadirachtin and sulphur (Siddiqui, 1992). The presence of sulphur might be responsible for maximum effect of neem oil than the other extracts. Fungitoxic properties of neem oil, cake and leaf extract on radial growth and biomass production in the present study have also been supported by Singh et al. (1980) who reported the inhibitory effect of neem oil against four pathogenic fungi. Singh et al. (1993) also observed the inhibition in radial growth of *S. rolfsii* by neem oil. Kazmi et al. (1995) and Ilyas et al. (1997) proved the efficacy of neem oil against several soil borne fungi including *M. phaseolina*. Shivpuri et al. (1997) reported the fungitoxic properties of neem leaf extract against several fungi. Aqueous extract of trunk bark though proved inhibitory for radial growth and biomass production of pathogen, it was relatively poor in performance when compared to other employed extracts.

Sclerotia are the primary unit of survival and play a major role in life cycle of *M. phaseolina* (Dubey and Upadhyaya, 2001). Effect of neem extract on survival of sclerotia of *M. phaseolina* was found inhibitory after 2 and 4 days of incubation. Sclerotia treated with neem oil did not germinate, hence resulted in 100% inhibition. The inhibition in germination and viability of *M. phaseolina* may be due to the presence of several bioactive compounds present in neem oil and cake extract. Dubey and Kumar (2003) have found the azadirachtin (30 ppm) as effective as the fungicide mancozeb after 72 h of treatment. Besides sulphur, neem oil contain a bitter yellowish substance which contain alkaloid, resins, glycosides and fatty acids. Sharma and Basandrai (1997) found leaf extract of *A. indica* effective in reducing sclerotial viability of *Sclerotinia sclerotiorum* isolated from beans.

Any amendment in the soil is ultimately subjected to decomposition by indigenous soil microorganism. During the course of decomposition there occurs evolution of some volatiles, which adversely affect the organisms. Fungistatic effects of volatile and non-volatile constituents of different neem extracts (5 %) were found inhibitory against sclerotial germination of *M. phaseolina*. Similar results were noticed for volatile fungistasis of oil. Effect of oil on sclerotial germination was increased with increase in concentration of oil and incubation period. Reduction in germination and viability of sclerotia of *M. phaseolina* may be attributed to the presence of volatile and non-volatile antifungal constituents in neem extracts. Among various fungistatic factors an important one is the increase in alkalinity of soil (Hora and Baker, 1974). Ammonia is evolved during the decomposition of neem oil and seed cake. Increase in pH of soil was found directly

propotional to ammonia production which in turn increased the fungistatic activity of soil (Hora and Baker, 1974). Mycostatic properties of ammonia have been reported mostly from alkaline soil. Volatile inhibitors from neem products may not kill or inactivate all the cells, hence the viable cells of sclerotia showed poor germination. The effect of volatile fungistasis was more pronounced than non volatile fungistasis. Dwivedi and Dubey (1987) also reported deleterious effect of volatile fractions of hydrodistillate of different neem products on germination of *M. phaseolina* sclerotia.

Thus, it may be concluded that the application of neem cake extract and neem oil posed high fungitoxic effects on *in vitro* radial growth and sclerotial survival of *M. phaseolina* in amended soil.

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Correspondence to:
Prof. R.C. Dubey
Department of Botany and Microbiology
Gurukul Kangri University
Haridwar- 249404
Uttarakhand, India
Phone: +91-1334-246767
Fax: +91-1334-246767

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