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SHORT COMMUNICATION

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Influence of growth medium on antifungal activity of neem oil (*Azadirachta indica*) against *Lagenidium giganteum* and *Metarhizium anisopliae*

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Abstract The inhibition of mycelial growth of *Lagenidium giganteum* by neem oil was lower than that of *Metarhizium anisopliae* in PYG and Emerson's YpSs agar media. However, neem oil did not inhibit the mycelial growth of *L. giganteum* in sunflower seed extract agar medium, but did it inhibit the mycelial growth of *M. anisopliae*. The minimum inhibitory concentration of neem oil for *L. giganteum* was higher than that for *M. anisopliae*. The minimum fungicidal concentration of neem oil in PYG medium was lower than in YpSs for both fungi. The spores of *L. giganteum* grown in SFE medium could be used with neem oil for vector control.

Key words *Azadirachta indica* · *Lagenidium giganteum* · *Metarhizium anisopliae* · Minimum fungicidal concentration · Minimum inhibitory concentration

Development of resistance in malaria vectors to a broad spectrum of insecticides is the main obstacle for the disease vector control program. An integrated approach for the control of mosquitoes and the diseases they transmit is a better option to circumvent the problems associated with the use of chemical insecticides (Lacey and Orr 1994). Among the alternatives, natural products and entomopathogenic fungi have proved their usefulness in vector control in many parts of the world. *Azadirachta indica* A. Juss. is commonly known as neem in India. It is found throughout the central dry zone and the Siwalik hills in India (National Research Council 1992). Parts of the neem plant have been reported to have antibacterial, antifungal, antimalarial, and anticancer effect (Siddiqui et al. 1992; Udeinya 1993; Kusamran et al. 1998). Neem-based pesticides are extensively used in agricultural practices and vector control in many parts of the tropical world (Mittal and Subbarao 2003). Neem oil was found to be a potential mosquito larvicide (Mittal et al. 1995; Vatandoost and Vaziri 2004; Okumu et al. 2007). The plant extracts contain phenols, flavonoids, quinines, tannins, alkaloids, saponins, and sterols and are biodegradable nontoxic products; hence, plant extracts are suitable for use in integrated vector control (IVC).

Neem oil emulsion in water was found to control the breeding of *Culex quinquefasciatus* Say, *Anopheles (An.) stephensi* Liston, and *Aedes aegpti* Linn in pools, basement tanks, and desert coolers for 2–3 weeks (Batra et al. 1998). Control of mosquito breeding has also been demonstrated in the field and in some confined habitats using neem oil in water and neem oil-coated wood scraps (Nagpal et al. 1995). The entomopathogenic fungi *Lagenidium giganteum* Couch, *Beauveria bassiana* (Bals.-Criv.) Vuill., and *Metarhizium anisopliae* (Metschn.) Sorokin are reported to be effective against mosquito larvae. *Lagenidium giganteum* is an obligatory fungal pathogen of mosquito larvae and was found to be successful in field trials (Teng et al. 2005). *M. anisopliae* spores impregnated on black cotton cloth surfaces were found to be effective in controlling adult *Anopheles gambiae* Giles mosquitoes in Tanzania (Scholte et al. 2005) and reduced the malaria parasite transmission potential of *An. stephensi* (Blanford et al. 2005). Lacey and Lacey (1990) stated that integrated pest management strategy for mosquito control, also known as IVC (integrated vector control), is an ecologically safe approach that may involve several complementary intervention methods used alone or in combination. In this article, we report the feasibility of use of mosquito pathogenic strains of *L. giganteum* and *M. anisopliae* in combination with neem oil as a possible approach for integrated vector control.

The fungal isolates of *L. giganteum* (MTCC 719) and *M. anisopliae* (MTCC 3210) used in this study were obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh, India. The culture of *L. giganteum* was maintained in PYG agar medium (peptone 1.25 g, yeast extract 1.25 g, glucose 3 g, agar 20 g, distilled water 1 l) and *M. anisopliae* in Emerson's YpSs agar medium (yeast extract 4 g, soluble starch

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Table 1. Comparison of IC_{50} (ppm ± SE) of neem oil for inhibition of mycelial growth in *Lagenidium giganteum* and *Metarhizium anisopliae* in three different media

Name of the fungus	PYG	Emerson's YpSs	Sunflower seed extract
Lagenidium giganteum	413.7 ± 16.74	187 ± 4.72	NI[a]
Metarhizium anisopliae	104.94 ± 3.65	78.43 ± 4.91	72 ± 2.48

IC_{50}, 50% inhibition of mycelial growth
PYG medium: peptone 1.25 g, yeast extract 1.25 g, glucose 3 g, agar 20 g, distilled water 1 l; YpSs medium: yeast extract 4 g, soluble starch 15 g, dipotassium hydrogen phosphate 1 g, magnesium sulfate 0.5 g, agar 20 g, distilled water 1 l)
[a] No inhibition (NI) was observed up to 800 ppm

15 g, dipotassium hydrogen phosphate 1 g, magnesium sulfate 0.5 g, agar 20 g, distilled water 1 l).

Petri dishes for mycelial growth assay were prepared by adding commercially available neem oil (manufactured by Shree Ambika Oil Cake Industries, Nadiad, Gujurat, India) to the medium at five concentrations. Previously prepared 100 ml medium was boiled and cooled to 50° ± 5°C for pouring agar plates for culturing the fungus. Sunflower seed extract (SFE) agar medium was prepared as per the protocol of Rueda and Axtell (1991). The aforementioned strains of *L. giganteum* and *M. anisopliae* were used for the mycelial growth inhibition assay. Spores of mother culture of *M. anisopliae* and *L. giganteum* were transferred to Petri dishes containing Emerson's YpSs and PYG agar medium, respectively. The Petri dishes with spores of these two fungi were left for culture of fresh colony in an incubator maintained at 25° ± 2°C for 7 days. After visually ascertaining the growth, small blocks of agar (~4 mm) from the outer edge of the colony having a sufficient number of spores and mycelia of *M. anisopliae* were transferred to Petri dishes containing Emerson's YpSs agar with neem oil at concentrations of 50, 100, 200, 400, and 800 ppm and a control Petri dish without neem oil. Five Petri dishes of fungus with these neem oil concentrations were prepared by adding appropriate amounts of neem oil in the agar medium before the media were poured into the Petri dishes. Two control Petri dishes were prepared for each replicate without neem oil. The Petri dishes were left in an incubator for 5 days at 25° ± 2°C for mycelial growth, and on day 5 the growth was assessed by measuring the diameter of each colony and compared with control. The percent inhibition in the mycelial growth in the Petri dishes with different concentrations of neem oil was calculated with reference to control as follows: percent inhibition = [1 − (growth in treated plate/ growth in control plate)] × 100.

A similar procedure was followed for the determination of inhibition by neem oil on mycelial growth of *M. anisopliae* in PYG and SFE media and *L. giganteum* in PYG, Emerson's YpSs, and SFE media. Experiments were carried out three times on different days.

The MIC is the lowest concentration of neem oil that inhibits the growth of the fungi, as was determined by Pyun and Shin (2006). To increase the miscibility of neem oil, 1 ml each dimethyl sulfoxide (DMSO) and Tween 80 was mixed with 98 ml neem oil. Neem oil was added to sterile nutrient liquid broth (PYG, Emerson's YpSs, and SFE) to make concentrations of 10, 20, 40, 80, 100, 125, 200, and 600 µl/ml. To 3.0 ml of sterile medium with neem oil, 0.5 ml of the exponentially growing fungal culture (containing $>10^4$ spores/ml) grown in PYG, Emerson's YpSs, and SFE agar media was added. One set of tubes containing only the growth medium and another set with growth medium, Tween 80, and DMSO were the two controls for the experiment. The tubes with fungal colonies were incubated at 25° ± 2°C for 24 h, and the growth was estimated by measuring the optical density at 520 nm in a spectrophotometer. The experiments were conducted three times on different days. The MIC in SFE broth could not be determined because of dense precipitation in the broth.

Table 2. Minimum inhibitory concentration (MIC) of neem oil (µl/ml) against *Lagenidium giganteum* and *Metarhizium anisopliae* in PYG and Emerson's media

Strain	MIC	
	PYG	Emerson's YpSs
Lagenidium giganteum	28 ± 5.29	21.64 ± 5.77
Metarhizium anisopliae	12.40 ± 2.51	11.34 ± 1.15

MFC is defined as the highest dilution (lowest concentration) at which no growth was observed on the Petri dishes. Tubes without fungal growth were confirmed by reculture on agar Petri dishes incubated at 25° ± 2°C for 48 h. Each experiment was performed three times on different days.

The concentration of neem oil required to inhibit 50% of mycelial growth compared to the control (without neem oil) is defined as IC_{50}; this was calculated by Log Probit regression analysis using software SPSS 9.0 (SPSS, Chicago, IL, USA).

In this present investigation, inhibition of mycelial growth and fungicidal activity of neem oil was observed in *L. giganteum* and *M. anisopliae*. The IC_{50} of neem oil for inhibition of *L. giganteum* and *M. anisopliae* is shown in Table 1. The inhibition of mycelial growth of *M. anisopliae* by neem oil was significantly different ($df = 2, 12$; $F = 8.26$, $P = 0.005$) in PYG, YpSs, and SFE agar media. Neem oil inhibited the mycelial growth significantly ($P = 0.002$) in the YpSs agar medium compared to PYG agar medium for both fungi (Table 1). Inhibition of the mycelial growth of *M. anisopliae* was significantly higher than that of *L. giganteum* in PYG ($P = 0.0003$) and YpSs agar medium ($P = 0.03$). The IC_{50} of the strains indicated that the mycelial growth of *L. giganteum* was 3.9- and 2.4 fold less than that of *M. anisopliae* in PYG and Emerson's YpSs media, respectively.

Table 3. Minimum fungicidal concentration (MFC) of neem oil (µl/ml) against *Lagenidium giganteum* and *Metarhizium anisopliae* in three different media

Strain	MFC		
	PYG	Emerson's YpSs	SFE
Lagenidium giganteum	59.32 ± 5.13	60 ± 5	OG[a]
Metarhizium anisopliae	42.76 ± 4.04	63.30 ± 2.80	41.66 ± 2.88

SFE, sunflower seed extract
[a] Growth observed up to 600 µl/ml neem oil

Mycelial growth of *L. giganteum* was not inhibited in SFE agar medium with neem oil, in contrast to *M. anisopliae*. MIC results of the neem oil against *L. giganteum* and *M. anisopliae* in PYG and Emerson's YpSs liquid media are shown in Table 2. The MIC of neem oil against *L. giganteum* was significantly higher ($P = 0.0009$) in PYG broth (28 ± 5.29 µl/ml) than YpSs broth (21.64 ± 5.77 µl/ml). The MIC of neem oil to *M. anisopliae* in PYG and Emerson's YpSs was not significantly different ($P = 0.15$). The MIC of *L. giganteum* was significantly higher than that of *M. anisopliae* in PYG ($P = 0.005$) and Emerson's YpSs ($P = 0.03$) broth, respectively. In both cases, neem oil was found relatively more effective in Emerson's YpSs medium than in PYG medium. The MFC of neem oil for *M. anisopliae* in Emerson's YpSs broth was significantly ($P = 0.0006$) higher than PYG broth (Table 3). However, the MFC of neem oil for *L. giganteum* was the same for both broths. The MFC of neem oil in PYG broth for both fungi was significantly different ($P = 0.012$), whereas for YpSs broth it was not significantly different ($P = 0.104$).

Studies on inhibition of mycelial growth in SFE medium at various concentrations of neem oil (50, 100, 200, 400, and 800 ppm) indicated that *L. giganteum* remained uninhibited, in contrast to *M. anisopliae*. Hirose et al. (2001) recorded 36% inhibition with neem oil on the mycelial growth of *B. bassiana* and *M. aniospliae*. Visalakshy et al. (2006) studied the compatibility of seven plant oils with the growth of *Paecilomyces farinosus* (Holmsk.) A.H.S. Br. & G. Sm. and observed maximum growth in sunflower oil and intermediate growth in the medium with neem oil.

The results of the two articles just mentioned showed that the effect of neem oil on mycelial growth of fungi was not specific and varied from species to species. The strains of *M. aniospliae* and *L. giganteum* used in this present investigation are mosquito pathogenic, and their compatibilities for use with neem oil were studied. The mycelial growth of *L. giganteum* was not influenced by neem oil (up to 800 ppm) in SFE medium, whereas the mycelial growth of *M. anisopliae* was inhibited in all three media (PYG, 104 ppm; Emerson's YpSs, 78.43 ppm; SFE, 72 ppm). These results indicated that *M. anisopliae* in combination with neem oil is not a feasible proposition for IVC. However, use of the entomopathogenic fungus *L. giganteum* in combination with neem oil in SFE medium could be a possible option for IVC.

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