Efficacy of neem leaf extract against it's own fungal endophyte *Curvularia lunata*

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The efficacy and in vitro activity of leaf extract of neem (*Azadirachta indica* A. Juss.) was carried out on an endophytic fungus *Curvularia lunata*, isolated from neem leaf. Results shows that different concentration variants (Viz. AzPDA 20, AzPDA 40, -- so on) of leaf extract significantly reduce the mycelial growth of *Curvularia lunata*, after 4 days of incubation. Maximum 78.8% inhibition of mycelial growth was recorded in AzPDA 60, after first day of incubation and least 14.6% was in AzPDA 20, after four days of incubation. However AzPDA 100 shows absolute inhibition for first day of incubation, but gradually decline to 43.9% on fourth day of incubation. It was observed in all concentration variants that during transition of second to third day there is significant increase in percentage inhibition of mycelial growth Viz. AzPDA 20 by 6.7%, AzPDA 40 by 6.4%, AzPDA 60 by 1.5%. Comparative analysis of all concentration variants however shows handsome net percentage inhibition, it was AzPDA 100 shows maximum 56.1% net percentage mycelial growth inhibition, while AzPDA 20 shows the least 35.4% net percentage inhibition.

Key words: endophytic fungi, *Azadirachta indica, Curvularia lunata*, and inhibitory activity.

Introduction

The neem tree (*Azadirachta indica* A. Juss) is a tropical evergreen plant native to east India and Burma. It grows in much of Southeast Asia and West Africa. In modern research concerns it gains equally high preference due to the potential of using neem derivatives such as leaf extract, oil, seed kernel extract, for preparation of environment friendly 'soft-herbicides'. Not only these conventional approaches limit the potential exploitation of the neem plant, but also some new fascinating areas were open for further research, and one of them is to explore the endophytic microbial diversity.

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Fungal endophytes: exploring hosts again

Endophytes are those microbes, which inhabit the living tissues without showing any overt clinical symptom of pathogenicity (Sturz *et al*., 2000, and Wilson, 1995). So far as many plants including palms, citrus, mango, banana, cashew etc. has been studies for endophytic mycoflora. Rodrigues and Samuals (1990) isolate endophytes from tropical palm of genera *Licuala ramasayi*, 11 fungi were isolated including an unusual isolate of *Fusarium aquaeductum.* Xylariaceous fungi were most frequent ones (Rodrigues, 1991). From Banana (*Musa acuminate)* 16 fungal taxa were isolated among which *Xylaria* spp. was most frequent genus, followed by *Colletotrichum musae* and *Cordana musae* (Pereira *et al.,* 1999). In Mango (*Mangifera indica*) several pathogenic fungi occurs as endophytes, prior to inflorescences emergence *Dothiorella* sp. And *Phomopsis mangifera* was found more frequents in trees not spread with copper. Endophytic colonization of inflorescences and pedicel tissues was consider to be a primary route of infection for fruits that develops rot stem end during ripening (Johnson *et al.,* 1992). Endophytic fungi from leaf of Cashew nut (*Annacardium occidentale*) growing in four Brazilian north eastern states 21 endophytic fungi were reported, *Colletotrichum gloeosporiodes, Pestalotia* spp.*, Fusarium solani* and *Phomopsis* sp. were the prominent endophytes. In Columbia, an endophytes *Phomopsis* sp. was isolated from the woody host *Cavendishia pubescens.* The fungus produce Paspalitrems A and C (Sutherland *et al*., 1999; Clay and Holah, 1999). In Uruguay the endophytic mycobiota of two species of Eucalyptus was characterized. The diversity of endophytes isolated was low, as was the number of host specific species (Bettuci, 1993).

Fungal endophytes from Azadirachta indica

Rajagopal and Suryanarayana have investigated the endophytic fungi of the leaves of Neem plant. Their study shows the effect of leaf tissue type and seasonality on endophytes assemblage and colonization. They recorded only *Fusarium avenaceam* along with some sterile mycelia (Rajagopal, and Suryanarayana, 2000). Few scattered studies are also done in the area but the work was not considered all aspects of *Azadirachta indica* as compares with its medicinal and therapeutic values. Some recent work furthermore added some new genera to the list (Mahesh *et al.,* 2005) according to them a total of 77 endophytes belonging to 15 genera were isolated from the inner bark of neem.

This present work however evaluates the efficacy of neem leaf extract against the endophyte *Curvularia lunata,* which was isolated from the neem itself. This is interesting because earlier workers has shown the efficacy of neem derivatives against various phytopathogenic fungi, but efforts were needed to evaluate the efficacy of neem leaf extract against it's own endophytic mycoflora. In this regard this is first ever attempt has been taken to evaluate the efficacy of neem leaf extracts against it's own endophytes.

Materials and methods

Isolation of endophytic fungi

Random samples from plant (*Azadirachta indica* A. Juss) containing asymptomatic leaves were taken. The samples were thoroughly washed in running tap water and then surface were sterilized by submerging them in 75 % ethanol for 2 minutes. The samples were further sterilized in to 5.3 % NaOCl (v/v) for one minute and there after dipped in to 75 % ethanol for 30 seconds. After drying in sterile condition small discs were cut and placed on PDA amended by 50 mg/l chloramphenicol to suppress bacterial contaminations. The Petri dishes were incubated for 25 days on 25 ± 2ºC in BOD cum humidity chamber. Leaving rest of the fungal genera *Curvularia lunata* was screened and maintained on fresh PDA plates.

Extraction of leaf Extract

Fresh mature leaves of neem plant were taken for preparing leaf extract. The leaves were thoroughly washed in running tap water followed by sterile distilled water, and fine slurry was prepared by taking 200 gm of leaf with 100 ml of sterile water. The slurry was filtered through the double fold muslin cloth, and the extract was kept as stock solution of extract (SE). This stock solution is then added in to sterilized PDA along with vancomycin, nystatin, ampicillin, and streptomycin sulfate each at 50 ppm concentration to avoid contaminations to amended plates. This amended PDA (AzPDA) is now poured in four different Petri plates, the toxicity of which was then determined at different concentrations, AzPDA 20, AzPDA 40, AzPDA 60 and AzPDA 100, by following the poisoned food technique.

Growth of mycelium

Each Petri plate with amended PDA was used for growth estimation of mycelium. The small inoculation discs of 5 days old culture of *Curvularia lunata* on PDA medium were placed in the centre of well-made extract amended PDA plates. The plates were incubated for 5 days at 27±1ºC. The average diameter of resultant colony was measured. The growth of *Curvularia lunata* mycelium on PDA without any amendment was used as control. The percentage inhibition of mycelial growth by the leaf extract was calculated by using the formula

$$\% \text{ Img} = (\text{CD} - \text{TD}) \times 100 / \text{CD}$$

Where, %Img = Percentage inhibition of mycelial growth
CD = Colony diameter of control plate
TD = Colony diameter of test plate

Results and Discussion

The efficacy of neem leaf extract is found very effective against test fungus *Curvularia lunata* (Table 1). For first day of incubation if we compared it against AzPDA20, it is 50% less, AzPDA40 nearly 57.1% less, AzPDA 60 78.8% less (Table 2). If we exempted AzPDA100 from the rest, it is AzPDA60 is more efficacious AzPDA60 favored then rest showing 78.8% inhibition i.e. 31.2% mycelial growth, while AzPDA40 favored 42.9% mycelial growth with 57.1% inhibition. AzPDA20 on the other hand facilitates highest 50% of mycelial growth. Second day of incubation period however lead to decrease in percentage inhibition (Table 3) AzPDA20 for – 27.3%, AzPDA40 for – 25.3%, AzPDA60 for – 33.4%, while AzPDA100 decrease to – 40.9 %. The highest mycelial growth was favored by AzPDA20 - 77.3% (22.7% inhibition), and lowest by AzPDA20 - 41.9% (59.1% inhibition), both AzPDA40 and AzPDA60 shows moderate enhancement in mycelial growth with percentage inhibition of 31.8 and 45.4%, respectively. The result shows that AzPDA100 is showing nearly absolute inhibition after 24 h of incubation.

The transition from second to third day however positively favors the mycelial growth except for those of AzPDA100, which was again suppress the mycelial growth by 0.3%, while AzPDA20 modulate mycelial growth to + 6.7 %, which was highest amongst the deferent variants, and AzPDA60 also positively modulates by + 1.5 stand lowest amongst the variants. Third day of incubation again get momentum by facilitating mycelial growth to 70.6% (29.4% inhibition) for AzPDA20, and 41.2% (58.8% inhibition) for

AzPDA100, rest two variants shows moderate enhancement in mycelial growth with percentage inhibition of 38.2 and 47.0 for AzPDA40 and AzPDA60 respectively.

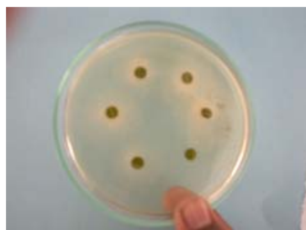

Fig. 1. Plate of 8 days of incubation shows emergency of endophytic mycelia (I did not found reference to this figure in text).

The percentage fluctuation for the third and fourth day of incubation, it was AzPDA100 shows highest fluctuation positive in favor of inhibition and lowest in favor of mycelial growth, while AzPDA60 shows lowest modulation in favor of mycelial inhibition and highest in favor of mycelial growth. On account of percentage mycelial growth inhibition over control on 4 days of incubation period it was AzPDA100 with 43.9% mycelial growth inhibition found more efficacious then AzPDA20 with 14.6% of mycelial inhibition.

Let conclude safely that the net percentage inhibition on account of percentage fluctuation of mycelial growth, it was AzPDA100 with – 56.1% net inhibition stands more promising to check the growth of *Curvularia lunata*, while the AzPDA40 with 32.8% net inhibition shows less efficacy for mycelial inhibition of *Curvularia lunata*.

What is remarkable is that increasing the toxicity of leaf extract the efficacy of mycelial inhibition was also enhanced proportionally. Despite of this fact that concerned fungus itself was isolated endophyticaly from neem plant the efficacy of neem leaf extract against it was striking. What did the author expect, that because the fungus resides in close proximity of neem constituents no significant response would receive? However result shows significant efficacy of neem leaf extract against *Curvularia lunata*, this means the fungal microbe either have any mechanism that enables them not to encountered by metabolic constituents of neem leaf in, *in vivo* conditions or if it encountered then how can it dissipate the effects of neem constituents. This unexplained and interesting aspects are left behind untouched for future workers.

Table 1. Effect of neem leaf extract on mycelial growth of *Curvularia lunata* after 4 days of incubation period.

Incubation Period (In Days)	Radial growth measurement (cm), after 4 days of incubation on medium*				
	AzPDA20	AzPDA40	AzPDA60	AzPDA100	control
1	0.7	0.6	0.3	0.1	1.4
2	1.7	1.5	1.2	0.9	2.2
3	2.4	2.1	1.8	1.6	2.4
4	3.5	3.1	2.6	2.3	4.1
Mean ±SD	1.97±1.02	1.82±1.05	1.47±0.97	1.22±0.94	2.77±1.20

*The toxicity was determined by the poisoned food technique.

Table 2. Percentage inhibition over control, after 4 days of incubation period.

PDA Variants (Extract amended)*	% Inhibition over control, after 4 days of incubation period			
	1	2	3	4
AzPDA20	50.0	22.7	29.4	14.6
AzPDA40	57.1	31.8	38.2	24.3
AzPDA60	78.8	45.4	47.0	36.5
AzPDA100	ND**	59.1	58.8	43.9

*The toxicity was determined by the poisoned food technique
**No significant mycelial growth was visualized during 24 h incubation.

Table 3. Percentage fluctuation over control, after 4 days of incubation period.

*PDA Variants (Extract amended)	% Inhibition over control, after 4 days of incubation period			
	1-2**	2-3	3-4	NET % inhibition
AzPDA20	-27.3	6.7	-14.8	-35.4
AzPDA40	-25.3	6.4	-13.9	-32.8
AzPDA60	-33.4	1.5	-10.5	-42.4
AzPDA100	-40.9	0.3***	-14.9	-56.1

*The toxicity was determined by the poisoned food technique
**The transition between first and second day. Similarly second and third day and third and fourth day.
***During second to third day transition AzPDA 100 still shows increase in inhibition while rest were in favor of mycelial growth.

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