Antifungal activity of essential oils on *Phomopsis azadirachtae* causing die-back of neem

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ABSTRACT

Phomopsis azadirachtae is a phytopathogenic fungus that causes die-back of neem. The aim of this study was to evaluate the antifungal activity of five essential oils on the growth of *Phomopsis azadirachtae* isolated from die-back infected neem twigs. The poisoned food method was used to test the antifungal activity of essential oils against *P. azadirachtae* at 0, 100, 200, 300 and 400 ppm concentrations. Except cinnamon oil all other oils tested *viz*., basil oil, camphor oil, lavender oil and rose oil completely inhibited the mycelial growth of the pathogen at 400 ppm concentration. At 300 ppm concentration all the five essentials oils inhibited the phytotoxin production by *P. azadirachtae*.

Keywords: Die-back of neem, *Phomopsis azadirachtae*, essential oil, fungitoxic, biocontrol

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INTRODUCTION

Neem (*Azadirachta indica* A. Juss) is a well-known medicinal plant across the globe for its medicinal, pesticidal, agricultural and industrial uses (Girish and Shankara Bhat, 2008a). Neem is a tropical evergreen tree native to Indian sub-continent and is an important multipurpose social forestry species. This tree is presently suffering from destructive die-back disease caused by *Phomopsis azadirachtae* Sateesh, Bhat and Devaki, a deuteromycetous fungus (Sateesh *et al*., 1997; Girish and Shankara Bhat, 2008b). This disease results in almost 100% loss of fruits production and drastic reduction in evergreen canopy thereby reducing the life expectancy and seed production (Girish *et al*., 2009a).

Fungal plant diseases are primarily managed by the application of synthetic fungicides (Maloy, 1993) and die-back of neem can also be managed by synthetic fungicides (Girish *et al*., 2009b). However, the use of synthetic fungicides needs to be reduced owing to their pollutive effects, non-biodegradability and residual toxicities. This has led to the search for the development of non-toxic, biodegradable and eco-friendly agents for the management of plant pathogens (Fathima *et al*., 2009).

Natural products from plants represent a rich source of alternative, environmentally more acceptable disease management agents (Habung Yami and Shukla, 2016). The advantages of using plant-derived antimicrobials in comparison to synthetic chemicals are their low toxicity, high degradability, multiple mechanisms of action and lesser side effects (Raja, 2014). Recently, there has been great interest in essential oils for controlling phytopathogens (Katooli *et al*., 2011; Gakuubi *et al*., 2017). Essential oils are major plant products having a broad spectrum of activities including biocidal activity against broad range of organisms such as bacteria, fungi, viruses, protozoa and insects (Kalemba and Kunicka, 2003). Essential oils are derived from aromatic plants that contain terpenes, aromatic and aliphatic compounds (especially alcohols, esters, ethers, aldehydes, ketones, lactones, phenols and phenol ethers) (Bakkali *et al*., 2008; Rana *et al*., 2011). Some of these compounds are capable of inhibiting fungal growth and numerous studies have documented the antifungal properties of many plant essential oils (Abdolahi *et al*., 2010; Habung Yami and Shukla, 2016). In the

Table 1. Essential oils tested against *Phomopsis azadirachtae* for antifungal activity

Essential oil	Plant source	Family	Plant part used
Basil oil	*Ocimum basilicum* L.	Lamiaceae	Leaves
Camphor oil	*Cinnamomum camphora* L.	Lauraceae	Chipped wood, root stumps and branches
Cinnamon oil	*Cinnamomum zeylanicum* Blume	Lauraceae	Bark and leaves
Lavender oil	*Lavandula angustifolia* Mill.	Lamiaceae	Flowers
Rose oil	*Rosa damascene* Mill.	Rosaceae	Flowers

present study, five essential oils of aromatic plants (Table 1) were screened *in vitro* for their antifungal activity against the die-back of neem pathogen.

MATERIALS AND METHODS

Isolation of *Phomopsis azadirachtae*

The infected twigs collected from die-back affected neem trees were cut in to 2 to 3 cm pieces including the middle transition region of healthy and infected portions using sterile blades. Twig pieces were washed with running tap water for an hour. Then they were cut into 1-1.5 cm segments with a transition zone at the middle portion. The bark was removed and the segments were washed thoroughly with running tap water and surface sterilized using sodium hypochlorite solution (with 5% available chlorine), then they were rinsed five times with sterile distilled water. The surface sterilized twig segments were placed in Petri plates containing potato dextrose agar (PDA, Himedia) amended with 100 ppm of chloramphenicol (20 ml / plate), aseptically at the rate of four segments per plate. The inoculated plates were incubated for 7 days at 26 ± 2^{o}C with 12 h photoperiod and observed for the growth of the pathogen from the twig segments. The incubation was continued for 15 days to allow sporulation. The spores were identified microscopically for the confirmation of the presence of *P. azadirachtae*. The isolated pathogen was sub-cultured on to fresh PDA plates by transferring 5 mm mycelia-agar disc drawn from the margin of mycelial mat of *P. azadirachtae* culture, incubated at 26 ± 2^{o}C with 12 h photoperiod for 7-days and were maintained at 4^{o}C until used.

Essential oils against *P. azadirachtae*

Five essential oils *viz*., basil oil, camphor oil, cinnamon oil, lavender oil and rose oil were used to study their effect on *P. azadirachtae* by poisoned-food technique (Fathima *et al*., 2009). Stock solution of 10,000 ppm concentration of each essential oil was prepared by dissolving in acetone. Stock solutions were added at different concentrations separately to sterile PDA to obtain concentrations of 100, 200, 300 and 400 ppm. The PDA medium with only acetone (400 ppm) served as control. The oil amended medium was poured into sterile 90 mm diameter Petri plates (20 ml / plate). Five mm mycelia-agar disc of *P. azadirachtae* obtained from the margin of seven-day-old culture was inoculated at the centre of both control and essential oil amended PDA plates. All the plates were incubated at 26 ± 2^{o}C with 12 h photoperiod. After 10 days of incubation the colony diameter was measured and sporulation was observed after 15 days of incubation. The per cent mycelial growth inhibition (PI) with respect to the control was computed using formula PI = (C-T / C) X 100 where C is the colony diameter of the control and T is that of the treated combinations. The experiment was repeated three times.

Essential oils against toxin production by *Phomopsis azadirachtae*

P. azadirachtae is reported to produce a phytotoxin that plays a role in its pathogenecity (Girish *et al*., 2009a). Based on the results of mycelial growth inhibition, 300 ppm concentration was selected for this study.

Stock solutions of each essential oil were added to sterile potato dextrose broth (PDB, Himedia) separately to obtain concentration of 300 ppm. PDB without oil was also maintained. 100 ml of both treated and untreated PDB in 250 ml Erlenmeyer flask was inoculated with 5 mm diameter mycelia-agar disc of *P. azadirachtae*. Totally 1.0 l of medium was inoculated. PDB that was untreated and uninoculated served as control.

All the flasks were incubated for 25 days at 26 ± 2°C with 12 h photoperiod. Then the culture broth was filtered using Whatman No. 1 filter paper and the culture filtrate of each treatment was collected separately. The culture filtrates thus obtained were filter-sterilized using 0.45 µm membrane filter discs. The extraction of crude toxin and neem seed treatment, with 500 and 1000 ppm of crude toxin extract, was carried out as per Girish *et al.* (2009a).

RESULTS AND DISCUSSION

Isolation of *Phomopsis azadirachtae*

The pathogen was isolated from the die-back infected neem twig samples and maintained on PDA plates (Fig. 1 & 2). The isolate was confirmed as *P. azadirachtae* by observing the mycelial nature, mycelial pigmentation and production of pycnidia and two types of conidia - alpha and beta conidia (Sateesh *et al*., 1997).

Figure 1. *Phomopsis azadirachtae* isolated from infected die-back neem twig on PDA

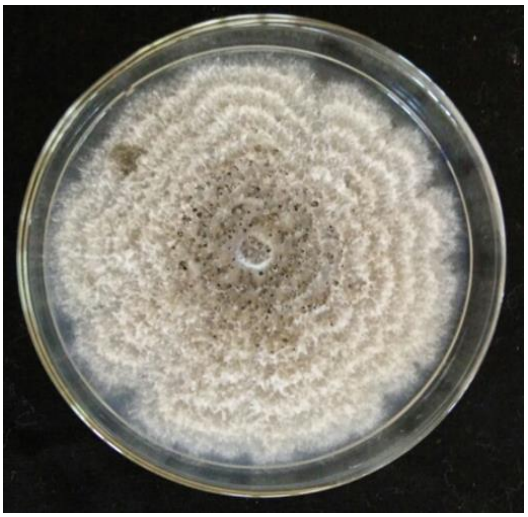

Figure 2. *Phomopsis azadirachtae* culture on PDA (10-days-old)

Essential oils against *P. azadirachtae*

All the five essential oils tested showed significant antifungal activity on *P. azadirachtae*. Essential oils of basil, camphor, lavender and rose were very effective in inhibiting the growth of *P. azadirachtae.* All these oils completely inhibited the mycelial growth of the pathogen at 400 ppm. Cinnamon oil showed moderate antifungal activity against *P. azadirachtae*. Though complete inhibition was not observed at the tested maximum concentration of 400 ppm, an increase in the percent inhibition with increase in the concentration of cinnamon oil from 100 to 400 ppm was observed (Table 2). Comparatively, rose oil showed the best activity followed by camphor oil, basil oil, lavender oil and least activity was observed with cinnamon oil.

Essential oils against toxin production by *P. azadirachtae*

In all the PDB flasks treated with 300 ppm of essential oils minimal mycelial growth was observed. Neem seeds treated with 500 ppm and 1000 ppm of crude toxin extracts from all the essential oils treated and *P. azadirachtae* inoculated broths showed seed germination similar to treatment with extract from control broth (without oil and pathogen inoculation). 500 ppm and 1000 ppm of crude toxin extracts from culture broth of *P. azadirachtae* without oil treatment completely inhibited the seed

Table 2. Effect of essential oils on the mycelial growth of *Phomopsis azadirachtae*

Concentrations of essential oil (ppm)	Colony diameter (in cm)				
	Basil oil	Camphor oil	Cinnamon oil	Lavender oil	Rose oil
0 (Control)	9.0 ± 0.21	7.9 ± 0.38	9.0 ± 0.21	9.0 ± 0.21	7. 69 ± 0.38
100	5.24 ± 0.08	4.96 ± 0.12	3.63 ± 0.12	6.9 ± 0.21	2.96 ± 0.12
200	3.15 ± 0.12	2.13 ± 0.16	3.2 ± 0.16	3.3 ± 0.08	1.15 ± 0.12
300	0.96 ± 0.08	0.79 ± 0.12	2.56 ± 0.20	1.06 ± 0.12	0.76 ± 0.12
400	0.0 ± 0.0	0.0 ± 0.0	2.20 ± 0.20	0.0 ± 0.0	0.0 ± 0.0

Values given are means of three replicates ± S.E.

Table 3. Effect of crude toxin extracts on the germination of neem seeds

Crude toxin extract from	Percent germination	
	500 ppm	1000 ppm
PDB (untreated with oil but *P. azadirachtae* inoculated)	0.0 ± 0.0	0.0 ± 0.0
PDB (untreated + uninoculated)	89.50 ± 0.30	88.75 ± 0.35
PDB + Basil oil (300 ppm) + *P. azadirachtae*	87.50 ± 0.42	87.25 ± 0.27
PDB + Camphor oil (300 ppm) + *P. azadirachtae*	87.50 ± 0.25	86.75 ± 0.31
PDB + Cinnamon oil (300 ppm) + *P. azadirachtae*	86.75 ± 0.42	86.25 ± 0.30
PDB + Lavender oil (300 ppm) + *P. azadirachtae*	88.75 ± 0.35	87.50 ± 0.30
PDB + Rose oil (300 ppm) + *P. azadirachtae*	88.75 ± 0.27	87.75 ± 0.42

Values given are means of four replicates ± S.E; PDB – Potato Dextrose Broth

germination and caused seed quality deterioration. These results indicated the absence of toxin in culture filtrates from essential oil treated broths and the presence of toxin in culture filtrate from untreated but inoculated broth (Table 3). Comparatively, based on seed germination, rose oil and lavender oil showed better activity than basil oil and camphor oil while cinnamon oil exhibited least activity.

This study indicated that plant essential oils possess antifungal activity and can be exploited as an ideal treatment for future plant disease management programs eliminating the fungal spread. All the five essential oils screened such as basil oil, camphor oil, cinnamon oil, lavender oil and rose oil significantly inhibited the mycelial growth of *P. azadirachtae in vitro*. *In vitro* assays are simple yet critical methods especially in preliminary screening of antimicrobial compounds (Girish *et al*., 2009b). The decrease in colony diameter or growth of fungus was in correspondence to the concentration of oil i.e. colony diameter decreased with the increase in concentration of essential oils.

Among the different concentrations of essential oils tested 300-400 ppm seemed to be most effective range. However, the levels of essential oils necessary to inhibit microbial growth in fields are generally higher than in culture media due to interactions between essential oil compounds and the environmental components (Nuchas and Tassou, 2000). In the case of cinnamon oil, a decrease in the fungal mycelial growth with increase in the concentration indicates that although complete inhibition has not been observed at the tested concentrations, higher concentrations might completely inhibit the growth of *P. azadirachtae*. From the results of seed germination studies it is evident that all the five essential oils at 300 ppm concentration, although allowed little mycelial growth, completely inhibited the production of phytotoxin by *P. azadirachtae*. Essential oils are reported to inhibit the production of toxins

by fungi (Sumalan *et al*., 2013; Gemeda *et al*., 2014).

Essential oils contain a high percentage of monoterpenes, eugenol, cinnamic aldehyde, thymol, terpenes and phenolic compounds. Antimicrobial activity could be correlated with these bioactive compounds present in essential oils (Sharma and Tripati, 2008; Rana *et al*., 2011). Essential oil can inhibit microorganisms by various mechanisms such as affecting the activities of membrane catalyzed enzymes, acting as uncoupling agents and interrupting ADP phosphorylation, interfering with membrane-integrated or associated enzymes by stopping their production or activity. Essential oils also inhibit the synthesis of DNA, RNA, proteins and polysaccharides in fungal and bacterial cells (Mohan, 2008). In fungi, they act on hyphae, causing loss of rigidity and integrity of the cellular wall of the hyphae, resulting in the death of mycelium (Sharma and Tripati, 2008). The inhibition of fungal growth observed in the present study with the increase in oil concentration may be attributed to some of the microbicidal and fungitoxic effects mentioned above.

The present study suggests that all the five essential oils tested *viz*., basil oil, camphor oil, cinnamon oil, lavender oil and rose oil possess antifungal potential under laboratory conditions and could be used for the eco-friendly management of *P. azadirachtae*. However, this is a preliminary investigation to know the potential of some essential oils against fungi. Further study is warranted to know the active compounds present in these oils and their mode of action. The results obtained from this study will form a basis for further investigations in this regard.

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