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Antifungal activity of essential oils against *Phomopsis azadirachtae*- the causative agent of die-back disease of neem

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Six essential oils Clove, Cedar wood, Lemon grass, Peppermint, Citronella and Nutmeg oils were tested for *in vitro* antifungal activity on *Phomopsis azadirachtae.* The test organism is the causative agent of destructive die-back disease of neem. Different essential oils were screened using food poisoning technique. All the used six oils showed significant antifungal activity against the tested pathogen. The results indicated that the citronella and lemongrass showed 100% inhibition of mycelial growth at 2,500 ppm. Hence, the results of the present investigations indicate that plant essential oils possess antifungal activity and can be exploited as an ideal treatment for future plant disease management to eliminate fungal spread.

Key words: Antifungal activity, *Azadirachtae indica*, essential oils, neem, *Phomopsis azadirachtae*,

Introduction

Effective management of a plant disease is a key to save plants from diseases caused from microbes, since plants are significant as they are both economical and aesthetic. Die-back of neem in one of the most destructive disease on neem which is spreading very rampantly in different parts of Karnataka and Tamilnadu (Nagendra Prasad *et al*., 2006, 2007a). Fungal plant diseases are usually controlled by application of fungicides (Maloy, 1993). Among the different management strategies used in controlling fungal plant diseases, chemicals rule the roost. However extensive use of chemicals as antifungal agents might lead to severe side effects such as carcinogenicity, terotogenecity, oncogenecity and other genotoxic properties (Basilico and Basilico, 1999). Further extensive use of chemicals leads to biohazordous

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effects on ecosystem. Further, their persistent applications lead to resistance in pathogens against these fungicides (Brent, 1995). Thus alternative approaches are preferred which are ecofriendly (Anandraj and Leela, 1996).

Essential oils from plant edible parts which are ecofriendly in nature have been used by several workers for controlling fungi, bacteria, viruses and insect pests (Singh and Upadhyaya, 1993; Singh, 1996). The main reasons for using essential oils as antifungal agents is their natural origin and low chance of pathogens developing resistance. The complexicity of essential oils are attributed to their terpene hydrocarbons and their oxygenated derivatives such as alcohols, aldehydes, ketones, acids and esters (Tzortazakis *et al.,* 2007). Antifungal properties of plant essential oils have been reported by researchers throughout world (Bouchra *et al.,* 2003; Daferera *et al.,* 2003). Even researchers in India have reported a number of essential oils for their antifungal activity (Paster *et al.,* 1995). A good number of essential oils are reported to be effective against many phytopathogenic fungi (Srivatsava and Singh, 2001).

Hence, in the present study, some important essential oils of aromatic plant species have been screened for their antifungal activity against the die-back pathogen of neem. The non-toxic, non-pollutive and biodegradable nature of these essential oils prompted to exploit these natural products of higher plants against *P. azadirachtae*.

Materials and methods

Revival of Phomopsis azadirachtae isolates

Phomopsis azadirachtae isolates maintained on MEA slants at 4° C after isolation from neem twig samples were freshly transferred on to Petri plates containing PDA and incubated at 26 °C$\pm$2 °C for 4 days.

Essential oils

Clove, Cedar wood, Lemon grass, Peppermint, Citronella and Nutmeg oils were tested for antifungal activity on *Phomopsis azadirachtae.* Six essential oils used in this study were obtained from Karnataka Aromas, essential oil distillery, Bangalore, Karnataka. They produce and commercially sell / export essential oils.

Determining the antifungal activity

Agar dilution method

Screening of essential oils for antifungal activity on *Phomopsis azadirachtae* was conducted using the agar dilution method. Each tested oil was used at different concentrations: 500, 1,000, 1,500, 2,000 and 2,500 ppm. The oils were completely dissolved in Tween 20 (S D Fine chemicals, Mumbai, India) and added to the 20 ml of PDA medium before solidification into Petri dish. One disc (0.5 cm diameter) of mycelial plug, taken from the edge of four to six day old fungal cultures, was transferred into the Petri dish. The plates were incubated in alternating periods of 12h darkness and 12h of light at 28±2 ^{0}C for 7 days. Controls consisted only Tween 20 mixed with PDA and were handled similarly with plates containing essential oils. The efficacy of treatments was evaluated from all the five plates by measuring fungal colony development (in cm). The percent mycelial growth inhibition (P) with respect to the control was computed from the formula as follows:

$$P = \frac{(C-T)}{C} \times 100$$

Where C is the colony diameter of the control and T is of that of the treated ones. The experiment contained five replications and was repeated three times.

Statistical analysis

The SPSS statistical methods [SPSS for windows (Version 10.2) were used to calculate the means, standard errors and standard deviations. Statistical analysis one-way ANOVA was applied to the data to determine differences. To check significant differences between the levels of the main factor, Tukey's multiple comparison tests at 5% significance was applied.

Results

The antifungal activity of clove, cedar wood, lemongrass, peppermint, citronella and nutmeg essential oils on *Phomopsis azadirachtae* growth in PDA and as well as statistical analysis (mean ± standard error) are shown in Table 1. The essential oils of citronella showed 100% inhibition on mycelial growth of *P. azadirachtae* (Fig. 1) at 1,500 ppm .Where as peppermint oil showed 85.5% and cedar wood oil 80.6% at the same concentration. Nutmeg oil and clove oil showed 79.1% and 78.8% respectively at the maximum concentration of essential oil tested. Lemon grass oil showed complete inhibition of mycelial growth of *P. azadirachtae* at 2,000 ppm concentration. Citronella oil provided

the highest degree of inhibition of *P. azadirachtae* growth because at the lowest concentration, it was found more potent inhibitor of growth than the other essential oils tested. It was observed that as the oil concentration increased the inhibitory effect increased. In other words, the inhibitory effect of the oil is proportional to its concentration.

Table 1. Antifungal activity of essential oils on the growth of *Phomopsis azadiracht*.

Conc. ppm	Colony diameter in cms ± S. D.					
	Nutmeg oil	**Peppermint oil**	**Clove oil**	**Citronella oil**	**Lemon grass oil**	**Cedarwood oil**
0	9.00± 0.00 [a*]	9.00± 0.00 [a]	9.00± 0.00 [a]	9.00± 0.00 [a]	9.00± 0.00 [a]	9.00± 0.00 [a]
500	9.00± 0.00 [b]	7.90±0.48 [b]	7.00±0.16 [b]	6.40±0.26 [b]	7.00±0.26 [b]	9.00± 0.00 [a]
1000	8.30± 0.24 [c]	6.40± 0.32 [c]	6.30± 0.22 [c]	4.40±0.24 [c]	5.60± 0.36 [c]	7.94±0.17
1500	6.20± 0.35 [d]	4.00± 0.36 [d]	5.90 ±0.16 [d]	0.00± 0.00 [d]	4.60± 0.72 [d]	6.06±0.13 [c]
2000	5.40±1.59 [e]	1.90 ±0.46 [e]	2.50± 0.20 [e]	0.00±0.00 [d]	0.00± 0.00	4.68±0.89 [d]
2500	1.88±0.54 [f]	1.30± 0.24 [f]	1.90 ±0.16 [f]	0.00±0.00 [d]	0.00± 0.00 [e]	1.74±0.51 [e]

*Figures followed by superscript of same letter in a column are not statistically significant (P = 0.05) according to Tukey's multiple-range test.

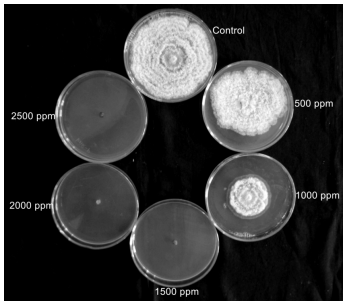

Fig.1. Effect of citronella oil on the mycelial growth of *Phomopsis azadirachtae*.

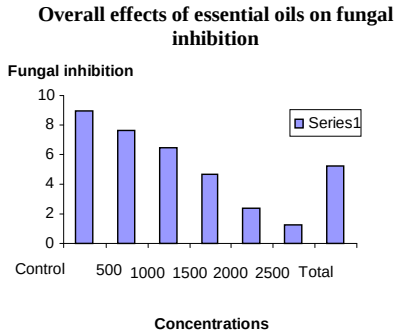

Fig. 2. Overall effect of Essential oils on mycelial inhibition of *Phomopsis azadirachtae.*

Discussion

The objective was to study the effect of essential oils on the mycelial growth of *P. azadirachtae*. The large scale application of synthetic fungicides has been cautioned due to their non-biodegradability, pollutive nature and residual toxicities. Chemicals also considered being deleterious for associated soil microbiota (Bunker and Mathur, 2001). Synthetic chemicals are also known to possess carcinogenic, teratogenic, oncogenic and genotoxic properties. Further many plant pathogens can develop resistance to synthetic fungicides with continuous exposure (Brent, 1995). Most of these chemical fungicides have been condemmened by environmentalists and are considered to be the most important man-made pollutant (Khoshoo, 1980). This has led to finding ecofriendly alternative approaches for management of plant diseases (Cook and Baker, 1983; Lyon *et al*., 1967; Ahmed *et al*., 1999; Parveen and Kumar, 2004). Softer biological measures for the control of plant diseases are gaining popularity in recent years Biocontrol agents are considered are better alternatives with different mechanisms of action than chemical pesticides.

A lot of researchers have documented the antimicrobial activity of essential oils including lemongrass, citronella, clove, peppermint, thyme and oregano oils against different fungal species (Mishra and Dubey, 1994; Viuda-martos *et al*., 2007). The present study has evaluated the effect of six essential oils on *P. azadirachtae* isolated from die-back affected neem twigs. The organism *Phomopsis azadirachtae* was confirmed as *Phomopsis azadirachtae* by traditional and molecular method (Nagendra Prasad *et al.*, 2007b; 2007c). Although all oils tested inhibited the growth of the fungus at different concentrations, citronella oil proved to be most potent inhibiting the mycelial growth completely at as low as 1,500 ppm. In the present study, two essential oils from lemon grass and citronella have given promising results against *P. azadirachtae*. These results confirm the antimicrobial activity of all the essential oils used in the present study. Citronella oil showed excellent growth inhibition of the fungus followed by the essential oils of lemongrass, peppermint, cedarwood, nutmeg and clove. Our results indicated the efficacy of citronella and lemon grass on the inhibition of fungal mycelium. *In vivo* studies should be performed to fully understand the overall process when citronella and lemongrass are sprayed on diseased neem trees. In general, the levels of essential oils and their compounds necessary to inhibit fungal growth are higher in practical condition than in culture media. This can be due to interaction between the phenolic compounds and other environmental factors (Nuchas and Tassou, 2000) and so, should be considered for commercial applications (Tzortzakis *et al*., 2007).

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 fungal spread. Overall effect of essential oils on mycelial inhibition of *P. azadirachtae* is as shown in the fig. 2. Among the different concentration of essential oils tested 2,000-2,500 ppm seems to be the most effective range. Recently, there has been great interest in essential oils from aromatic plants for controlling plant pathogens (Soliman and Badeaa, 2002; Valero and Salmeron, 2003). The information obtained in the present study suggests that essential oils show promising results in controlling the growth of *P. azadirachtae* under laboratory conditions.

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