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Fungi toxic efficiency of some plant volatile essential oils against plant pathogenic fungi

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Plant essential oils have the potential to replace the synthetic fungicides in the management of different fungal diseases. Four different essential oils of eucalyptus (*Eucalyptus globulus*), citronella (*Cymbopogon citrate*), karanj (*Pongamia pinnata*) and neem (*Azadirachta indica*) were selected because of their high inhibitory activities against ten phytopathogenic fungi. The antifungal screening for all the four Eos clearly indicates the effective lowest concentration to control the fungal growth. The more lower the MIC value, the better antifungal potency of the relative plant volatile *E. globulus*. Among the plant volatile essential oils, eucalyptus oil showed the lowest minimum inhibitory concentration (MIC), *i.e.* 0.5 mg/disc. Higher MIC values were registered, in the order, for citronella, karanj and neem oils, singly used to control all the ten fungal pathogens. Highest zone of inhibition (ZI) values followed the same pattern. These results indicated that plant volatile essential oils after suitable formulation could be used to control of different fungal pathogens. This may encourage the farmers to produce organic commodities to generate more revenue.

Key words Essential oils, minimum inhibitory concentration (MIC), zone of inhibition, fungal disease.

INTRODUCTION

Essential oils (Eos) may be defined as volatile oils that may be obtained from plant materials by steam distillation (Guenther, 1949). In the last few years there has been an increasing interest in Eos as substitutes for conventional synthetic pesticides. This has been due, in part, to concerns over pollution, the development of resistance to conventional pesticides (Holmes and Eckert, 2009), and to the needs of producers of organic agricultural products.

Certain parts of various plant species harbour secondary metabolites, which show a variety of chemical structures (Bell and Charlwood, 1980). Their roles are mostly unknown though many of them have been found to exhibit anti-fungal properties (Patel and Jasrai, 2009; Sujatha, 2010). Various types of anti-fungal chemicals such as saponins, unsaturated lactones, cyanogenic glycosides, oils and phenolic compounds are found to be present in relatively large quantities in tissues of some

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Table 1. CFU count of ten different fungal pathogen under experiment.

Fungus	Host plant	Plant parts	Incubation (days)	CFU
Fusarium equiseti (Fe)	brinjal	Root	3	1.36×10^6
Colletotrichum gloeosporioides (Cg)	brinjal	Leaf	6	2.64×10^6
Alternaria alternata (Aa)	tomato	Leaf	4	2.11×10^6
Pestalotiopsis theae (Pt)	tea	Leaf	7	3.45×10^6
Aspergillus flavus (Af)	ground nut	Pod	5	1.54×10^6
Fusarium solani (Fs)	potato	Root	3	1.67×10^6
Alternaria solani (Ac)	mustard	Leaf	5	2.82×10^6
Bipolaris oryzae (Bo)	rice	Leaf	7	0.97×10^6
Erysiphe pisi (Ep)	pea	Leaf	4	1.98×10^6
Cercospora nicotianae (Cn)	tobacco	Leaf	8	2.65×10^6

Table 2. Chemical compound present in the selected plant volatile essential oils.

Volatile oil source	Chemical compound
Eucalyptus leaves	1,8-cineole, eucalyptol, ursolic acid, globulol, geranyl acetate, terpinolene, limonene, epiglobulol, p-cymene, camphene, pinocarveol. A and β pinene, α-phellandrene, β-sabinene, α-terpineol, α-terpineol acetate
Citronella leaves	Myrcene, citral, citronellal, cymbopogonol, limonene, citronellic acid, citronellal, citronellol and geraniol
Karanj leaves	Saponins, hydroxyl furanoflavones, beta sitosterol glucoside and aurantiamide acetate, triterpenes, isoflavonoid diglycosides
Neem leaves	Linoleic acid, Oleic acid, 9-Hexadecenoic acid, Alpha-linolenic acid, Methyl isoheptadecanoate, 2,6,10,14-Tetramethylheptadecane, Hexadecanoic acid, Octadecanoic acid

*source: Pawar and Thaker (2007).

plant species. Their occurrence, distribution and possible functions have been reviewed by Schlosser (1988). Anti-fungal action of Eos and other chemical plant components has been reported by several scientists (Mann and Markhan, 2006; Deena and Thopil, 2008; Demirci et al., 2009; Mathpal et al., 2005 and Simic et al., 2004).

Considering the environmental pollution, we should exploit different plant volatile Eos for disease management in organic system (Barman et al., 2015). Keeping the value of synthetic pesticide free organic commodity, *in vitro* study was carried out. In this study, the antifungal potency of four different plant volatile Eos from eucalyptus (*Eucalyptus globulus*), citronella (*Cymbopogon citrate*), karanj (*Pongamia pinnata*) and neem (*Azadirachta indica*) has been tested against ten selected plant pathogenic fungal isolates and results achieved are presented.

MATERIALS AND METHODS

This experiment was conducted during the year 2012 to 2013. Distilled Eos (Upshaw Aromatics Private Ltd. Hyderabad) of four plant species, namely eucalyptus, citronella, karanj and neem were purchased from the local market, and were screened for *in-vitro* antifungal activity against the following 10 phytopathogenic fungi (Table 1) isolated from infected material: *Fusarium equiseti*, *Colletotrichum gloeosporioides*, *Alternaria alternata*, *Pestalotiopsis theae*, *Aspergillus flavus*, *Fusarium solani*, *Alternaria solani*, *Bipolaris oryzae*, *Erysiphe pisi*, and *Cercospora nicotianae* grown in Sabouraud Dextrose Agar (SDA) medium and maintained in the laboratory at 25±1°C.

The pathogens were subjected to Koch's postulates for verification of the diseases. Thereafter, they were incubated at 4°C. After seven days of incubation, the hyphal tip of the fungus radiating from the infected tissue was transferred onto SDA slants. Freshly prepared sterile SDA slants were used for the maintenance of the fungal cultures by sub-culturing periodically. Pathogens grown on sterile SDA media were stored in two different conditions, viz. at low temperature in refrigerator (at 4°C) and in incubator at 27±1°C. At the interval of one week, subculture was done taking sample from incubator at 27±1°C for preparation of inoculums for different experiments.

To avoid loss of virulence, fresh isolations were made when required. The chemical compounds present in the selected plant volatile Eos are listed in Table 2. The antifungal screening was performed through Disk diffusion assay method (Patel and Jasrai, 2011).

Paper disc preparation for the assay

Sterilized Whatman paper (No.1) discs (6.5 mm in diameter) were impregnated with the known quantity of plant volatile EO at 0.5 to 8 mg/disc, and then air dried. The impregnated discs were used to conduct bioefficacy study against the above mentioned fungal isolates.

Inoculum preparation of test fungi

50 µl of each fungal culture from SDA slant was transferred and established into a 150 ml conical flask containing 25 ml of SDA

Table 3. Essential oil minimum inhibitory concentration (MIC) values determined through Disc-diffusion assay method towards the selected fungi.

Volatile oil source	Inhibition of fungi at MIC value (mg/disc)					
	0.5	1.0	2.0	3.5	5.0	8.0
Eucalyptus	Af, Fs	Fe, Aa	Cg, Ac	Ep, Bo, Pt,	Cn	-
Citronella	-	Ac, Aa	Bo, Ep	Fe, Fs, Af	Cg, Cn	-
karanj	-	Cn, Cg	Pt, Aa	Af, Bo, Ac	Ep, Fe, Fs	-
Neem	-	Aa	Af, Cg	Ep, Pt	Ac, Bo, Fe, Fs	Cn

*Fusarium equiseti (Fe), Colletotrichum gloeosporioides (Cg), Alternaria alternata (Aa), Pestalotiopsis theae (Pt), Aspergillus flavus (Af), Fusarium solani (Fs), Alternaria solani (Ac), Bipolaris oryzae (Bo), Erysiphe pisi (Ep), Cercospora nicotianae (Cn).

Table 4. Zone of inhibition (ZI) against fungi exhibited by eucalyptus (*Eucalyptus globulus*) volatile oil at various concentrations in Disc-diffusion assay method.

Fungal phytopathogen	Zone of inhibition (mm) at different essential oil concentration (mg/disc)					
	0.5	1.0	2.0	3.5	5.0	8.0
Fusarium equiseti	-	3.44	4.67	8.48	10.45	12.37
Colletotrichum gloeosporioides	-	-	5.14	7.59	11.23	12.64
Alternaria alternata	-	4.12	5.39	6.78	9.78	11.34
Pestalotiopsis theae	-	-	-	6.45	8.68	10.98
Aspergillus flavus	-	4.31	6.54	8.45	10.28	13.88
Fusarium solani	3.89	4.37	6.98	8.59	11.67	14.45
Alternaria solani	-	-	4.69	5.67	9.58	13.54
Bipolaris oryzae	-	-	-	5.98	9.57	11.25
Erysiphe pisi	-	-	-	5.78	7.78	9.56
Cercospora nicotianae	-	-	-	-	4.12	6.48

medium for bioassay study. The inoculated flasks were successively incubated for a specific time period at room temperature (25±1°C). The number of all fungal colonies/flask was recorded by means of a haemocytometer spore count (Table 1). Then the fungal colonies formed in each single flask were homogenized in sterile conditions and the relative suspensions were used in the bio-assay study (Patel and Jasrai, 2011).

Evaluation of antifungal activity

Fungitoxic spectrum of the selected plant volatile Eos was determined at various concentrations (0.5 to 8 mg/disc) using the standardized protocol of the Disk diffusion assay (Patel and Jasrai, 2011) under axenic conditions. For this, an aliquot 0.1 ml fungal culture of known (spore count) unit forming colonies (UFC) was aseptically transferred with micropipette in each Petri plate containing SDA medium (15 ml in a 3.5 cm thick layer), and uniformly seeded on its surface with sterilized cotton swabs (Himedia). At the same time, extract loaded single Whatman paper discs were placed and slightly pressed on the media surface with sterile forceps to ascertain a firm contact. Then the plates were incubated in upside-down position for 72 h at 25±1°C. The experiment was performed in triplicate with untreated controls. The Zone of inhibition (ZI) indicating the EO antifungal effectiveness was measured (in mm) by the antibiotic Zone reader (Labfine) (Patel and Jasrai, 2011). The experiment was carried at five different concentration viz. 0.5, 1.0, 2.0, 3.5, 5.0 and 8.0 mg/disc. Three replications were maintained for each pathogen with CRD design. The data were subjected to statistical analysis using INDOSTAT package developed by Indostat service Hyderabad, India.

RESULTS AND DISCUSSION

Results of this study showed that the four tested plant volatile Eos had excellent broad- spectrum antifungal activity against the ten selected plant fungal pathogens. The inhibition of fungal phytopathogens by the tested Eos can be due to presence of complex mixture of secondary metabolites containing different volatile compounds such as phenylpropanes, various terpenoids and their oxygenated derivatives. The fungi toxic spectrum or MIC (minimum inhibitory concentration) values of the tested Eos, determined in terms of zone of inhibition (ZI), is presented in Tables 4, 5, 6 and 7. The antifungal screening for all the four Eos clearly indicates the effective lowest concentration to control the fungal growth. The more lower the MIC value, the better antifungal potency of the relative plant volatile EO. The lowest MIC value, 0.5 mg/disc, was recorded for EO from *C. citrate*. *P. pinnata* and *A. indica* EOs showed the maximum MIC values. *A. indica* EO showed minimum inhibition of fungal pathogen at certain MIC level (Table 3).

Table 5. Zone of inhibition (ZI) against fungi exhibited by citronella (*Cymbopogon citrate*) volatile oil at various concentrations in Disc diffusion assay method.

Fungal pathogen	Zone of Inhibition (mm) at different volatile oil concentration (mg/disc)					
	0.5	1.0	2.0	3.5	5.0	8.0
Fusarium equiseti	-	-	-	4.45	6.47	8.84
Colletotrichum gloeosporioides	-	-	-	-	4.25	6.58
Alternaria alternata	-	5.23	7.54	9.25	11.02	13.84
Pestalotiopsis theae	-	4.25	6.34	8.45	9.45	12.08
Aspergillus flavus	-	-	-	4.87	5.27	8.58
Fusarium solani	-	-	-	6.78	8.57	10.22
Alternaria solani	-	4.57	5.65	6.89	7.98	9.65
Bipolaris oryzae	-	-	5.97	7.35	9.56	12.35
Erysiphe pisi	-	-	4.89	6.58	8.69	10.91
Cercospora nicotianae	-	-	-	-	4.34	6.23

Table 6. Zone of inhibition (ZI) against fungi exhibited by karanj (*Pongamia pinnata*) volatile oil at various concentrations in Disc-diffusion assay method.

Fungal pathogen	Zone of inhibition (mm) at different volatile oil concentration (mg/disc)					
	0.5	1.0	2.0	3.5	5.0	8.0
Fusarium equiseti	-	-	-	-	4.87	6.78
Colletotrichum gloeosporioides	-	5.17	6.12	8.27	9.27	11.67
Alternaria alternata	-	-	6.24	7.98	8.94	11.28
Pestalotiopsis theae	-	-	6.78	8.37	10.45	12.98
Aspergillus flavus	-	-	-	4.65	6.58	7.98
Fusarium solani	-	-	-	-	4.78	6.25
Alternaria solani	-	-	-	4.15	6.78	11.28
Bipolaris oryzae	-	-	-	4.57	6.78	8.49
Erysiphe pisi	-	-	-	-	4.87	6.87
Cercospora nicotianae	-	4.26	5.67	7.89	9.45	11.34

Table 7. Zone of inhibition (ZI) against fungi exhibited by neem (*Azadirachta indica*) oil at various concentrations in Disc-diffusion assay method.

Fungal pathogen	Zone of inhibition (mm) at different volatile oil concentration (mg/disc)					
	0.5	1.0	2.0	3.5	5.0	8.0
Fusarium equiseti	-	-	-	-	4.35	6.98
Colletotrichum gloeosporioides	-	-	5.18	7.45	9.45	11.85
Alternaria alternata	-	4.25	6.57	8.48	10.78	12.98
Pestalotiopsis theae	-	-	-	4.75	6.45	9.48
Aspergillus flavus	-	-	4.57	6.25	8.49	10.95
Fusarium solani	-	-	-	-	4.08	6.94
Alternaria solani	-	-	-	-	4.02	7.03
Bipolaris oryzae	-	-	-	-	4.67	7.15
Erysiphe pisi	-	-	-	4.91	6.45	8.47
Cercospora nicotianae	-	-	-	-	-	4.83

In the present study, *E. globulus* EO exhibited highest zone of inhibition against *F. solani* (14.45 mm) followed

by *A. flavus* (13.88 mm). Its lowest MIC, 0.5 mg/disc, was recorded against *F. solani* followed by inhibition of *Aspergillus flavus*. The same EO at only 0.5 mg/disc was able to control *F. solani*. *C. citrate* EO showed maximum ZI against *A. alternata* (13.84 mm). *P. theae* was inhibited only at concentration of 1 mg/disc (Table 5). In contrast, *P. pinnata* EO successfully inhibited all tested fungi and exhibited its maximum ZI against *A. alternata* (12.98 mm) followed by *C. gloeosporioides* (11.67 mm).

In addition, the assay revealed that *P. pinnata* restricted the growth of all tested fungi at MIC of 5.0 mg/disc (Table 6). *A. indica* EO demonstrated highest ZI against *A. alternata* (12.98 mm) followed by *C. gloeosporioides* (11.85 mm), *A. flavus* (10.95 mm). But *A. indica* EO did not performe well in comparison to the other Eos (Table 7). All the selected fungi were inhibited with all the Eos even if at different performance levels. Fungal growth inhibition by Eos often involves prevention of hyphal growth and sporulation, interruption in nutrient uptake and metabolism, plasma membrane disruption, mitochondrial structure disorganization and interference with respiratory enzymatic reactions of the mitochondrial membrane (Patel and Jasrai, 2011).

Conclusions

Locally available plant volatile Eos may play a great role in controlling major plant disease. This may encourage the farmers to produce organic commodities to generate more revenue. As EOs antifungal activity is very probably due to the synergistic action of chemical compound mixtures, there would be a negligible chance of resistance development in fungal pathogens. Disease control through such natural available volatile substances would also be an important tool for integrated disease management in organic farming.

Conflict of Interests

The authors have not declared any conflict of interests.

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