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Fungitoxic evaluation of volatile oil and its active constituent against fungal pathogens of paddy plants

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Abstract
Background: Angiospermic plants have been recognized as the store house of several biologically active substances. They also store and emit volatile substances like essential oils which diffuse out in the vicinity. There are enough references to believe that essential oils, extracted from plants have enough antimicrobial potentialities and at the same time are largely non-toxic to humans as compared to synthetic antimicrobial substances. Besides being antimicrobial in nature, such substances are generally biodegradable, non- pollutive and can easily be procured.
Objective: The present project was undertaken to evaluate the fungitoxic efficacy of the volatile essential oil of *Myristica fragrans* and its active constituent 'Eugenol' against fungal pathogens of paddy plants viz., *Rhizoctonia solani*, *Pyricularia oryzae* and *Cochliobolus miyabeanus*in order to explore the possibility of their use as an alternative to synthetic fungicides. Various fungitoxic properties like nature of fungitoxicity, effect of temperature and storage on the antifungal efficacy of the volatile essential oil and its active constituent are also studied.
Materials and Methods: The volatile essential oil was extracted from sterilized dried arils of *Myristica fragrans* by hydro-distillation process in a Clevenger's apparatus. The fungitoxicity of the essential oil and its active constituent against test fungi was determined by Inverted Petri-Plate Technique.
Results: The volatile essential oil and its active constituent 'Eugenol'showed strong toxicity against test fungi. The toxicity of the oil did not change when subjected to high temperature conditions and variable periods of storage. Both volatile oil and its active constituent showed a wide range of antifungal spectrum.
Conclusion: The study, thus clearly reveals that the volatile essential oil of *Myristica fragrans* and its active constituent 'Eugenol' have strong fungitoxic activity and can be used to control fungal diseases of paddy plants.

Keywords: fungitoxicity, *Rhizoctonia solani*, *Pyricularia oryzae* and *Cochliobolus miyabeanus*, *Myristica fragrans*

Introduction

Seed treating chemical fungicides especially organo-mercurials are very costly and cause serious problems. They reduce the quality of crops, produce toxic effect on non-target organisms cause environmental pollution and resistance in pests and disease agents [13]. Therefore, these have become a popular target of conservationalists and are treated to be one of the most vital man-made pollutants. Considering all these dangerous effects, the control strategies are now directed towards replacing the use of hazardous chemical fungicides by environmentally friendly natural products [19].

The higher plants contain a wide variety of secondary metabolites having fungitoxic properties. Plant bio-chemicals especially volatile essential oils have also been reported to have antifungal properties against plant pathogens[1, 11]. The volatile essential oils are the by-products of plant metabolism and are commonly referred to as volatile plant secondary metabolites [15]. The use of volatile oils is regarded as the best suited ecofriendly measure as these are easily biodegradable and safer. During recent years many volatile oils have been proved to possess fungitoxic efficacy [6, 7, 12, 22, 24].

In the present communication project was undertaken to evaluate the fungitoxic efficacy of the volatile oil of *Myristica fragrans* and its active constituent 'Eugenol' against *Rhizoctonia solani*, *Pyricularia oryzae* and *Cochliobolus miyabeanus*in order to explore the possibility of their use as an alternative to organo-mercularials. Our findings on the various fungitoxic properties like nature of fungitoxicity, effect of temperature and storage on the antifungal efficacy of the volatile oil of *Myristica fragrans* and its active constituent are reported.

Materials and Methods

Culture of test fungi and their growth conditions

The pure cultures of *Rhizoctonia solani* Kuhn. (MTCC No. 4633), *Pyricularia oryzae* (MTCC No. 1477) and *Cochliobolus miyabeanus* (MTCC No. 2114) were procured from Microbial Type Culture (MTCC), Chandigarh. This fungus was grown on PDA plate at 27°C ± 2°C and maintained with periodic sub-culturing at 4°C. All these fungi are pathogens of paddy plants.

Isolation of essential oil

The dried aril of *Myristica fragrans* (nutmeg) were procured from the local market. 300 hundred gram of seeds was surface sterilized, dipping in 2.5 percent sodium hypochlorite solution (NaOCl solution) for 5 minutes. These were then thoroughly washed with sterilized water and finally chopped into pieces before subjecting to hydro-distillation in Clevenger's apparatus for 8 to 10 hrs. The water immiscible fractions of hydro-distillates were collected as volatile essential oils.

In vitro study of fungitoxicity of volatile oil and its active constituent against test fungi

The fungitoxicity of volatile oil and its active constituent against test fungi was determined by Inverted Plate Technique. 10 ml of molten PDA medium was poured in a pre-sterilized Petri plate. Chloramphenicol was added to the medium to prevent bacterial growth [11]. A fungal disc (5 mm in diameter) cut from the periphery of 7 days old culture of test fungus, with the help of flame sterilized cork borer and placed in the centre of the medium served as inoculum.

The different concentrations of volatile oil of *Myristica fragrans* and its active constituent 'Eugenol' were prepared separately. For treatment sets, the filter paper discs (5 mm in diameter) were impregnated with the desired concentration of the volatile oil or active constituent 'Eugenol' and placed in the centre of the petriplate lid. The petriplate then kept inverted so that the fungal disc got exposed to the fumes of volatile oil. For control sets, the same volume of distilled water was used as assay discs. The plates were incubated for 7 days at 27 ± 2°C. Colony diameters in mutual perpendicular directions were measured on the seventh day in assay plates. Fungitoxicity was recorded in terms of the percent inhibition of mycelial growth and calculated using the following formula (Singh and Tripathi, 1999).

$$\text{Percent inhibition of mycelial growth} = \frac{dc - dt}{dc} \times 100$$

Where

dc-Average diameter of fungal colony in control sets.

dt-Average diameter of fungal colony in treatment sets.

Minimum Inhibitory Concentration (MIC) of volatile oil and its active constituent

MIC is the concentration of volatile oil or its active constituent, required for absolute inhibition of mycelial growth of the test fungi. For this, the different concentrations of volatile oil (0.8×10^3µl/l, 0.9×10^3µl/l, 1.0×10^3µl/l, 1.5×10^3µl/l, 2.0×10^3µl/l, 2.5×10^3µl/l and 3.0×10^3µl/l) were prepared. In the similar way the different concentrations of active constituent 'Eugenol' (0.6×10^3µl/l, 0.7×10^3 µl/l, 0.8×10^3µl/l, 0.9×10^3µl/l and 1.0×10^3µl/l) were prepared. The MIC against *Rhizoctonia solani*, *Pyricularia oryzae* and *Cochliobolus miyabeanus* was determined by the Inverted Petriplate Technique as described above.

Effect of temperature on fungitoxicity

Five ml of volatile oil was taken in glass vials which is closed tightly and exposed to the different temperatures from 20^0C to 55^0C in electrical oven for an hour. The vials were then allowed to cool down to room temperature. Petriplates containing test fungus were exposed to the filter paper disc impregnated with heated volatile oil and incubated at 27 ± 2°C. The fungitoxicity of volatile oil was assessed by Inverted plate technique as described earlier.

Effect of storage on fungitoxicity

The volatile oil was stored for different time periods. Petriplates containing test fungus was exposed to filter paper disc impregnated with stored volatile oil. Petriplates were incubated at 27 ± 2°C and recorded on seventh day of incubation (Table 3). The fungitoxicity of volatile oil was determined by Inverted plate technique as described earlier.

Antifungal spectrum of volatile oil and its active constituent

The range of Fungitoxic efficacy of the volatile oil of *Myristica fragrans* Houtt. at 1×10^3 µl/l (v/v) concentration and its active constituent 'Eugenol' at 0.8×10^3 µl/l (v/v) concentration, was tested against twenty fungal pathogens *viz.*, *Aspergillus flavus* Link., *Aspergillus fumigatus* Fres., *Aspergillus nidulans* Wintage, *Aspergillus niger* van Tiegh., *Aspergillus ochraceus* Wilhelm, *Cladosporium herbarum* (Pers.) Link., *Curvularia geniculata* (Tracy & Earle) Boedijn, *Curvularia lunata* (Wakker) Boedijn, *Drechslerasativa* Bugn., *Epicoccum nigrum* Link, *Fusarium acuminatum* Ellis & Everhart, *F. avenaceum* Schl., *Fusarium oxysporum* Schlecht., *Macrophomina phaseoli* (Maublanc) Ashby, *Nigrosporaoryzae* (Sacc.) Mason, *Penicillium chrysogenum* (Thom.), *Pythium aphanidermatum*, *Sclerotium oryzae* Catt., *Sclerotium rolfsi* Saccardo. Petriplates containing test fungus were exposed to the filter paper disc impregnated with desired concentration of volatile oil or its active constituent and incubated at 27 ± 2°C. The fungitoxicity was assessed by Inverted plate technique as described earlier.

Result

The fungitoxic efficacy of volatile oils of ten plants was evaluated by Inverted petriplate technique. The results were recorded in Table - 1. The result revealed that out of ten plants, the volatile oil of *Myristica fragrans* Houtt. showed 100% inhibition of mycelial growth, followed by *Ocimum basilicum*(82%) and *Pavonia odorata* Wild. (80%).

The volatile oil of *Myristica fragrans* Houtt. and its active constituent 'Eugenol' were subjected for Minimum Inhibitory Concentration (MIC). The results were recorded in table (2) and table (3). The volatile oil of *Myristica fragrans* Houtt. completely inhibited the mycelial growth of *Rhizoctonia solani* and *Cochliobolus miyabeanus* at 0.9×10^3 µl/l concentration whereas *Pyricularia oryzae* at 1.5×10^3 µl/l concentration (Table - 2). The volatile oil was fungistatic at 1.5×10^3 µl/l concentration whereas fungicidal at 2.5×10^3 µl/l concentration. The active constituent 'Eugenol' of *Myristica fragrans* oil showed MIC at 0.7×10^3 µl/l for *Rhizoctonia solani* and *Cochliobolus miyabeanus* whereas at 0.8×10^3 µl/l concentration for *Pyricularia oryzae* (Table - 3). The study revealed that the volatile oil of *Myristica fragrans* Houtt. retained its fungitoxicity when it was exposed up to 50^0C temperature, however when they were exposed above than 50^0C temperature there was significant loss in their fungitoxic potential (Table - 4). When the volatile oil was stored for different periods of time, it retained their fungitoxic potential up to 365 days (Table - 4). The range of Fungitoxic efficacy of the volatile oil of *Myristica fragrans* Houtt. at 1×10^3 µl/l (v/v) concentration was tested against twenty fungal pathogens (Table- 5). Besides test fungi, the volatile oil inhibited completely the mycelial growth of eight other fungal pathogens *viz.*, *A. nidulans*, *A. niger*, *Cladosporium herbarum*, *Fusarium accuminatum*, *F. oxysporum*, *Macrophomina phaseoli*, *Sclerotium oryzae* and *S. rolfsi* (Table- 5). The active constituent 'Eugenol'at 1×10^3 µl/l (v/v) concentration, inhibited completely the mycelial growth of twelve fungal pathogens *viz.*, *A. nidulans*, *A. niger*, *Cladosporium herbarum*, *Curvularia geniculata*, *C. lunata*, *Fusarium accuminatum*, *F. avenaceum*, *F. oxysporum*, *Macrophomina phaseoli*, *Pythium aphanidermatum Sclerotium oryzae* and *S. rolfsi* (Table- 6). Both volatile oil and active constituent also showed moderate to strong toxicity to other fungi tested.

Discussion

Higher plants have been recognized as reservoirs of various biologically active substances [4, 25]. The substances extracted from plants are largely non- toxic to humans and at the same time have enough antimicrobial potentialities [3]. Besides being antimicrobial in nature, such substances are generally biodegradable[21] and non- pollutive [17] and can easily be procured from our largest renewable resources. There are enough references to believe that higher plants emit volatile substances like volatile essential oils which diffuse out in the vicinity and kill the pathogens, therefore these volatile oils have been used as fungicides for centuries to control plant diseases [20] in the recent years several workers reported volatile oils from various angiospermic plants have to possess fungitoxicity [8, 16, 26, 24, 28]. In the present study, the fungitoxicity of volatile oil of *Myristica fragrans* Houtt. of family Myristicaceae, is being reported here against *Rhizoctonia solani*, *Pyricularia oryzae* and *Cochliobolus miyabeanus* the common fungal pathogens of paddy plants. According to Wellman [29] the efficacy of a good fungicide must not be affected by extremes of temperature and long storage duration. The fungitoxicity of *Myristica fragrans* volatile oil was not affected by temperature treatment up to 50 °C and storage up to 365 days indicating thereby that the oil possesses a possibility of being a good fungicide. The volatile oil of *Myristica fragrans* in the present investigation possessed a broad range of fungitoxic spectrum which is a pre- requisite for successful employment of a fungitoxicant. Earlier workers have also reported broad range of fungitoxicity of different oils [9, 14, 24]. The fungitoxic efficacy of the volatile oils is due to presence of chemically active constituents [17, 27]. Tripathi *et. al.,* [17] isolated thymol and p-cymene as fungitoxic factors of *Trachyspermum* oil and recorded their fungitoxicity against *Aspergillus flavus* and *A. niger*. Singh *et. al.* [23] isolated dimethoxy agerato-chromine from the essential oil of *Ageratum* which possessed toxicity against dermatophytes. Dubey *et. al.*, [10] reported Geraniol as active antifungal principle oil of *Zanthoxylum alatum.* Barkai-Golan, [2] isolated and identified citral, limetin, 5-geranosy-7- methoxy coumarin and isopimpeneyin antifungal compounds from lemon peel. Bouzenna & Krichen, [5] reported citronellol as the active antifungal principle of *Pelargonium graveolens* essential oil against *Rhizoctonia solani*. In the present study 'Eugenol' is reported as active antifungal constituent from the volatile oil of *Myristica fragrans* against *Rhizoctonia solani*, *Pyricularia oryzae* and *Cochliobolus miyabeanus.*

The present investigation, thus clearly reveals that the volatile oil of *Myristica fragrans* and its active constituent 'Eugenol' may prove the ideal fungicides to control diseases caused by *Rhizoctonia solani*, *Pyricularia oryzae* and *Cochliobolus miyabeanus.* However *in vivo* investigations are pre- requisite before making any commitment regarding their practical utility as fungicides. The volatile oil appears to be non- toxic to human beings especially in view of the fact the parts exploited for isolating the oil is already in intimate human use.

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Conflict of Interest

The author declares that there is no conflict of interest.

Authors ' Contribution

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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None

Data Availability

All datasets generated or analyzed during this study are included in the manuscript.

Ethics Statement

This article does not contain any studies with human participants or animals performed by any of the authors.

Table 1: Fungitoxic screening of various volatile oils against test fungi

Essential oil tested	Part used	Yield (%)	Percent inhibition of mycelial growth								
			Rhizoctonia solani			*Pyricularia oryzae*			*Cochliobolus miyabeanus*		
			1×10³ µl/l	2×10³µl/l	3×10³µl/l	1×10³µl/l	2×10³µl/l	3×10³µl/l	1×10³µl/l	2×10³µl/l	3×10³ µl/l
Aegelmarmelos(L.) Corr./ (Rutaceae)	L.	0.2	19	35	75	18	35	65	21	41	56
Ageratum conyzoides (L.)/ Rh.(Asteraceae)	Rh.	0.02	25	40	45	25	38	48	22	35	43
Alpinia carinata (L.)Rh.(Zingiberaceae)	Rh.	0.3	19	42	68	20	42	71	20	38	60
Carum carvi (L.) (Apiaceae)	Fr.	0.8	15	31	61	15	30	48	19	35	65
*Cedrusdeodara**(Roxb.) Loud (Pinaceae)	W.	0.2	21	28	54	21	28	65	23	40	60
Curcuma amada(Roxb.) (Zingiberaceae)	Rh.	0.9	25	29	35	29	32	48	21	36	45
Lantana indica (Roxb.) (Verbenaceae)	L.	0.5	20	22	53	20	28	56	18	30	58
*Myristica fragrans*Houtt. (Myristicaceae)	S	100	100	100	100	95	100	100	100	100	100
Ocimum basilicum(Labiateae)	L.	0.4	28	68	82	28	63	85	26	65	82
Pavonia odorata Wild. (Malvaceae)	R.	0.2	23	68	80	28	66	88	25	56	80
	L: Leaves R: Roots Rh: Rhizome S: Seeds Fr: Fruits W: Wood chips*: Procured from market										

Table 2: Minimum Inhibitory Concentration (MIC) of volatileoil of *Myristica fragrans*Houtt.

Doses of oil used (µl/l)	Percent inhibition of mycelial growth		
	Rhizoctonia solani	***Pyricularia oryzae***	***Cochliobolus miyabeanus***
3.0×10^3	100	100	100
2.5×10^{3}**	100	100	100
2.0×10^3	100	100	100
1.5×10^{3}*	100	100	100
1.0×10^3	100	95	100
0.9×10^3	100	86	100
0.8×10^3	90	78	93
	**: Fungicidal, * : Fungistatic		

Table 3: Minimum Inhibitory Concentration (MIC) of active constituent 'Eugenol'

Doses (µl/l)	Percent inhibition of mycelial growth		
	Rhizoctonia solani	***Pyricularia oryzae***	***Cochliobolus miyabeanus***
1.0×10^3	100	100	100
0.9×10^3	100	100	100
0.8×10^3	100	100*	100
0.7×10^3	100*	92	100*
0.6×10^3	86	78	82
*: Fungistatic nature			

Table 4: Effect of temperature & storage on fungitoxicity of volatile oil of *Myristica fragrans* Houtt.

Parameter used		Percent inhibition of mycelial growth		
		Rhizoctonia solani	***Pyricularia oryzae***	***Cochliobolus miyabeanus***
Temperature treatment (°C)	20	100	100	100
	25	100	100	100
	30	100	100	100
	35	100	100	100
	40	100	100	100
	45	100	100	100
	50	100	100	100
	55	00	00	00
Storage (in days)	60	100	100	100
	120	100	100	100
	180	100	100	100
	240	100	100	100
	300	100	100	100
	365	100	100	100

Table 5: Antifungal spectrum of volatile oil of *Myristica fragrans* Houtt.at 1×103µl/l dose (v/v)

	Fungal species	**Percent inhibition of mycelialgrowth**
1.	*Aspergillus flavus* Link.	78
2.	*A. fumigatus* Fres.	82
3.	*A. nidulans* Wintage	100
4.	*A. niger* Van Tiegh	100
5.	*A. ochraceus* Wilhelm	45
6.	*Chaetomium indicum* Corda	70
7.	*Cladosporium herbarum* (Pers.) Link	100
8.	*Curvularia geniculata* (Traccy & Earle) Boedijn	95
9.	*C. lunata* (Wakker) Boedijn	90
10.	*Drechslera sativa* Bugn.	62
11.	*Epicoccum nigrum* Link	90
12.	*Fusarium accuminatum* Elis and Everhart	100
13.	*F. avenaceum* Schl.	92
14.	*F. oxysporum* Schlecht	100
15.	*Macrophomina phaseoli* (Maublanc) Ashby	100
16.	*Nigrospora oryzae*(Sacc.) Mason	68
17.	*Penicillium chrysogenum* (Thom.)	55
18.	*Pythium aphanidermatum*	95
19.	*Sclerotium oryzae* Catt.	100
20.	*S. rolfsi* Saccardo	100

Table 6: Antifungal spectrum of active constituent 'Eugenol'at 0.8×103µl/l(v/v)

	Fungal species	**Percent inhibition of mycelialgrowth**
1.	*Aspergillus flavus* Link.	90
2.	*A. fumigatus* Fres.	95

3.	*A. nidulans* Wintage	100
4.	*A. niger* Van Tiegh	100
5.	*A. ochraceus* Wilhelm	52
6.	*Chaetomium indicum* Corda	95
7.	*Cladosporium herbarum* (Pers.) Link	100
8.	*Curvularia geniculata* (Traccy & Earle) Boedijn	100
9.	*C. lunata* (Wakker) Boedijn	100
10.	*Drechslera sativa* Bugn.	65
11.	*Epicoccum nigrum* Link	90
12.	*Fusarium accuminatum* Elis and Everhart	100
13.	*F. avenaceum* Schl.	100
14.	*F. oxysporum* Schlecht	100
15.	*Macrophomina phaseoli* (Maublanc) Ashby	100
16.	*Nigrospora oryzae*(Sacc.) Mason	82
17.	*Penicillium chrysogenum* (Thom.)	72
18.	*Pythium aphanidermatum*	100
19.	*Sclerotium oryzae* Catt.	100
20.	*S. rolfsi* Saccardo	100

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