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Antifungal Activity of Essential Oils from *Eucalyptus citriodora* Hook. and *Cymbopogon citrates* (DC) Stapf. Against *Fusarium moniliforme* Sheld. Isolated from *Oryza sativa* Linn.

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Abstract

Antifungal activity of essential oils from *Eucalyptus citriodora* Hook. and *Cymbopogon citratus* (DC) Stapf. against *Fusarium moniliforme* Sheld. was studied by analyzing the mycelial growth of test fungus in different concentrations of essential oils within 10, 15 and 20 days. The infected sample of rice plants were collected from NARC and *Fusarium moniliforme* was isolated through blotter test method. The essential oils were extracted through hydro- distillation process using Clevenger oil extracting apparatus and were diluted with 95% ethanol and distilled water giving concentration of 1.2, 2.5, 3.7, 4.9, 6.2, and 12.4 µl ml^{-1} for *in -vitro* treatment. The Minimum Inhibitory Concentration (MIC)was 6.2 µl ml^{-1} for *Eucalyptus citriodora* and 4.9 µl ml^{-1} for *Cymbopogon citratus*. Both the oil were therefore significantly ($p < 0.05$, LSD = 5.41) effective to arrest the mycelium growth of the test fungus. Furthermore the negative correlations between the colony size of the test fungus and oil concentrations clearly supports the antifungal activity of these oils. Thus from comparative analysis of both the oils it was concluded that the oil of *Cymbopogon citratus* is more effective than the oil of *Eucalyptus citriodora.*

Keywords: *Foot rot, Hydro-distillation, In -vitro treatment, Minimum inhibitory concentration (MIC), Mycelial growth*

Introduction

The foot rot of rice caused by *Fusarium moniliforme* Sheld.was first reported from Italy in 1877. In Japan the disease was first described in 1898 and was known as "Bakanae disease". Foot rotor elongation disease is widespread in many rice growing areas in both tropical and temperate regions. The disease affects the host mainly in seedling stage and the symptoms are clearly seen in the nursery but causalities may occur throughout the life of the crop. Sometimes, the grain fails to germinate or the seedlings fail to emerge above the soil. The most conspicuous and detectable symptoms of the disease appear in the seedlings. Generally, the disease is seed borne and sometimes may be soil borne. Soil temperature and soil moisture influence the intensity of disease. The optimum temperature for the survival of pathogen lies between 25-30°C and excess of nitrogenous manures increase the intensity of disease.The pathogen is world- wide in distribution and also parasitizes the other graminaceous hosts such as sorghum, maize and sugarcane. The hyphae are slender, 3-5 µ broad, closely septate and much branched. Each micro-conidium is 1-2 celled, elliptic to ovate or oval in shape and measures 5-12 x 2-4 µ. The macro-conidiumis falcate, narrow at both ends 2-5 celled and measure 30-50 x 3 µ. They are formed singly or more often in clusters. The chlamydospores are produced rarely. The perfect stage is reported as *Gibberella fujikuroi* whose perithecia are superficial, globose, dark brown and measuring 270-350 x 240-300 µ. The clavate asci are formed in the perithecia. The ascospores are long ellipsoid, one-septa and measure 10-20 x 4-9 micron. Each ascus contains 4 or 6 ascospores (Pandey, 2003).

Two plants (*Eucalyptus citriodora* and *Cymbopogon citratus*)were taken in experiments. These plants are easily available aromatic plants in Nepal. These aromatic plants are very good source of essential oils which are mixture of different volatile aromatic compounds and can be extracted by steam or hydro-distillation from source plants. Main components from *Eucalyptus citriodora* plant reported are Citronnellal- 66%, Citronnellol-12%, Citronnellyl acetate- 4%, Isopulegol- 3% from 86% oxygenated compound (Fandohan et al. 2004)where as Myrcene-

28%, Neral (Citral B) - 20%, Geranial (Citral A) - 27%, Geraniol- 4% from 61% oxygenated compound are the main components of *Cymbopogon citratus*.

Methods and Materials

a. *Extraction of essential oils*

The essential oils of *Eucalyptus citriodora* and *Cymbopogon* citratus were extracted from their leaves by hydro-distillation method using Clevenger's oil extracting apparatus.

The oil collected was then dehydrated over anhydrous sodium sulphate and stored at 10°C.

b. *Media preparation*

Potato Agar Dextrose (PDA) media was prepared for culture of test fungal pathogen.

c. *Obtaining pure culture of test pathogen*

The fungus was obtained from infected leaves of Rice through Blotter Test Method. The pathogen was then identified by seeing and comparing their microscopic characters using the standard book by Booth, 1971. The pathogen from sample was then taken and inoculated into Petri-dishes containing PDA media and was incubated at 25° C with 12 hours of photoperiod. The pure culture of pathogen was thus obtained after 7 days.

Experiment

The toxicity of essential oil was assessed by using the Poisoned Food Technique given by Grover and Moore (1962) in whom the antifungal efficacy of oil was tested by poisoning the media with the oil.

The oils were tested at different concentration of 1.2, 2.5, 3.7, 4.2, 6.2 and 12.4 $\mu l ml^{-1}$ in PDA media to control growth of *Fusarium moniliforme.* These concentrations were obtained by diluting 20, 40, 60, 80, 100 and 200 µl of each oil in 100µl of ethanol plus 1ml of water and mixing with 15ml of melted sterile PDA. Each concentration of oil was poured into separate Petri-dishes with three replicas and mixed with 15ml of PDA media. Each Petri-dish was then inoculated at centre by a 5 mm diameter fungal disk taken from the rim of a seventh day old culture of test fungus. The inoculated Petri-dishes were incubated for 20 days at 25°C. Three Petri-dishes containing mixture of 1ml distilled water and 100 µl (95% ethanol) were inoculated to serve as control. Fungal growth was assessed by measuring colony diameter along two lines at right angles to each other at $10^{th.}$ $15^{th.}$ and $20^{th.}$ days. Average of these two measurements was taken as a single data for colony diameter.

Calculations

Fungal toxicity of essential oil was assessed in terms of percentage inhibition of mycelial growth of the test fungus (Rao & Srivastava, 1994).

$$\% \text{ inhibition of mycelia growth} = \frac{g_c - g_t}{g_c} \times 100$$

Where,g_c = growth of mycelia colony after incubation in control set and g_t= growth of mycelial colony after incubation in treatment set.

MIC was determined by the minimum concentration of oil required for 100%inhibition of mycelial growth of test fungus (Rao & Srivastava, 1994).

Statistical test

A factorial research design was adopted. ANOVA analysis was also carried out to find out the level of significance. The degree of probability ($P < 0.05$ or $P > 0.05$) has been incorporated into figures. Correlation analysis was carried out to find the relations between two variables where necessary. SPSS11.5 windows version was used for analytical statistics.

Results and Discussion

The activity of *Eucalyptus* and *Cymbopogon* oil against *Fusarium moniliforme* was analyzed by measuring the colony size at varying concentrations of essential oils in 10^{th}, 15^{th}, and 20^{th} days of incubation. The Eucalyptus oil at and above 6.2 µl ml^{-1} and Lemon grass oil at and above 4.9 µl ml^{-1}inhibited the mycelial growth of test fungus completely from $10^{th.}$ day of incubation. No growth

of test fungus at and beyond the 6.2 µl ml^{-1} concentration of each oil was noticed even after 15th. and 20th. days of incubations. The inhibition in the development of colony size of test fungus was increased along with increase in concentration of both essential oils. However the gradual increase in the size of a particular colony in each particular concentration, except minimum inhibitory concentrations (MIC) was also noticed during the observation of 10th. to 20th. days. Percent inhibition in colony size of the test fungus due to *Eucalyptus* oil at 10th,15th and 20th day of incubation is shown in Table no.1 ,2 and 3 respectively. whereas Percent inhibition in colony size of the test fungus due to *Cymbopogon* oil at 10th ,15th and 20th day of incubation is shown in Table no.4, 5 and 6 respectively. Similarly, the comparative effects for % inhibition in varying concentrations of *Eucalyptus* and *Cymbopogon* oil at10, 15 and 20 days of incubation are shown in the figure 1and 2 respectively.

The reported effective concentrations of *Eucalyptus citriodora* and *Cymbopogon citratusi.e.* 6.2 and 4.9 µl ml^{-1} respectively resembles to the effective concentration range of these essential oils given by Pattnaik et al.,1996 *i.e.* 0.25 – 10 µl ml^{-1}. The inhibitory effect of Eucalyptus oil against *Fusarium moniliforme* as noticed in this experiment is also supported by Rai et al., (1999). They also reported marked inhibition in fungal growth of *Fusarium* in *in vitro* conditions. Besides, the inhibitory role of *Cymbopogon citratus* at concentration 4.9 µl ml^{-1}, in this work is lower than that proposed by Fandohan et al., 2004. They proposed 8 µl ml^{-1} concentration for complete inhibition on the growth of same pathogen in corn. More effectiveness of *Cymbopogon citratus* than *Eucalyptus citriodora* for antifungal activities against *Fusariummoniliforme* in this research is also supported by Baruah et al., 1996 with almost same findings. The reason for antifungal activities of both essential oils may be attributed to their chemical compositions.

The comparative graph shown on figure 1 and 2 suggests that the *Cymbopogon* oil is effective than *Eucalyptus* oil at their similar concentrations. Furthermore, ANOVA suggests that the treatments are significant whereas the sources are not significant at 5% level. This indicates that irrespective of the sources of essential oils, their concentrations are effective in decreasing the mycelial growth of test fungus.

Table 1: Colony size on 10th. day of incubation in varying concentration of Eucalyptus oil.

S.N.	Inoculum size (mm)	Oil Con.n (µl ml^{-1})	Colony size (mm)			Mean colony size (mm)	Inhibition of mycelial growth (%)
			I	II	III		
1	5	0	30	29	32	30.33	0
2	5	1.2	22	21	24	22.33	26.37
3	5	2.5	17	18	19	18	40.65
4	5	3.7	15	14	15	14.66	51.66
5	5	4.9	7	8	9	8	73.62
6	5	6.2	5	5	5	5	100
7	5	12.4	5	5	5	5	100

Table 2: Colony size on 15th. day of incubation in varying concentration of *Eucalyptus* oil.

S.N.	Inoculum size (mm)	Oil Con.n(µl ml^{-1})	Colony size (mm)			Mean colony size (mm)	Inhibition of mycelial growth (%)
			I	II	III		
1	5	0	43	41	45	43	0
2	5	1.2	36	35	39	36.66	14.62
3	5	2.5	27	26	28	27	37.2
4	5	3.7	20	20	20	20	53.4
5	5	4.9	8.5	9.5	9	9	79.06
6	5	6.2	5	5	5	5	100
7	5	12.4	5	5	5	5	100

Table 3: Colony size on 20th. day of incubation in varying concentration of *Eucalyptus* oil.

S.N.	Inoculum size (mm)	Oil Con.^{n} (µl ml^{-1})	Colony size (mm)			Mean colony size (mm)	Inhibition of mycelial growth (%)
			I	II	III		
1	5	0	55	54	58	55.66	0
2	5	1.2	38	37	37	37.33	32.9
3	5	2.5	28	27.5	27	27.5	50.59
4	5	3.7	22	22	22	22	60.47
5	5	4.9	10	10.5	9.5	10	82.03
6	5	6.2	5	5	5	5	100
7	5	12.4	5	5	5	5	100

Table 4: Colony size on 10th. day of incubation in varying concentration of *Cymbopogon* oil.

S.N.	Inoculum size (mm)	Oil Con.^{n} (µl ml^{-1})	Colony size(mm)			Mean colony size (mm)	Inhibition of mycelial growth (%)
			I	II	III		
1	5	0	30	29	32	30.33	0
2	5	1.2	21	21	21	21	30.76
3	5	2.5	14	13	16	14.33	51.66
4	5	3.7	6.5	7	7.5	7	76.92
5	5	4.9	5	5	5	5	100
6	5	6.2	5	5	5	5	100
7	5	12.4	5	5	5	5	100

Table 5: Colony size on 15th. day of incubation in varying concentrationof *Cymbopogon* oil.

S.N.	Inoculum size (mm)	Oil Con.^{n} (µl ml^{-1})	Colony size(mm)			Mean colony size (mm)	Inhibition of mycelial growth (%)
			I	II	III		
1	5	0	43	41	45	43	0
2	5	1.2	34	34	35	34.3	20.2
3	5	2.5	25	24	26	25	41.8
4	5	3.7	7	7.5	7	7.2	83.3
5	5	4.9	5	5	5	5	100
6	5	6.2	5	5	5	5	100
7	5	12.4	5	5	5	5	100

Table 6: colony size on 20th day of incubation in varying concentration of cymbopogon oil.

S.N.	Inoculum size (mm)	Oil Con.^{n} (µl ml^{-1})	Colony size(mm)			Mean colony size (mm)	Inhibition of mycelial growth (%)
			I	II	III		
1	5	0	55	54	58	55.66	0
2	5	1.2	35	35	35	35	37.15
3	5	2.5	25	25	26	25.33	54.49
4	5	3.7	10	10	12	10.66	80.84
5	5	4.9	5	5	5	5	100
6	5	6.2	5	5	5	5	100
7	5	12.4	5	5	5	5	100

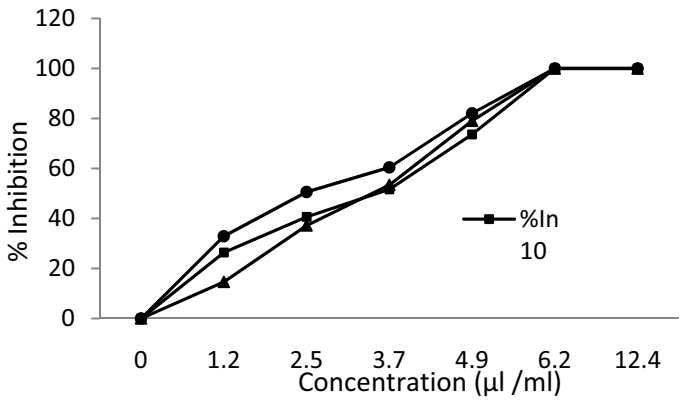

Figure 1: Comparative graph of % inhibition in varying concentrations of *Eucalyptus* oil at10, 15 and 20 days of incubation.

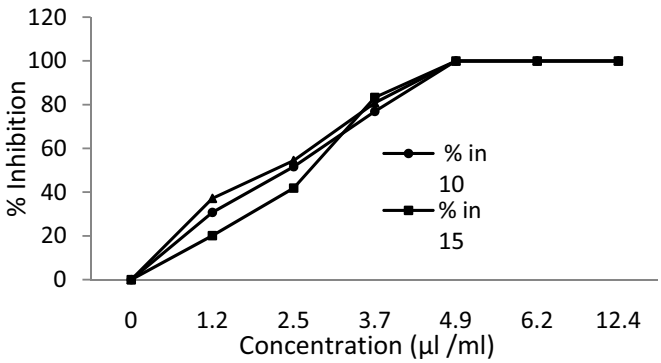

Figure 2: Comparative graph of % inhibition in varying concentrations of *Cymbopogon* oil at10, 15 and 20 days of incubation.

Hence, the essential oils used significantly ($p < 0.05$) inhibit the mycelial growth of the fungus irrespective of their sources. Effect of plant sources were however, not significant ($p > 0.05$). The concentration response was found as 12.4 µl ml^{-1} = 6.2 µl ml^{-1}> 4.9 µl ml^{-1}> 3.7 µl ml^{-1}> 2.5 µl ml^{-1}> 1.2 µl ml^{-1}>> 0 µl ml^{-1} with LSD value 5.42. Therefore, increasing the concentrations of essential oil there is gradual decrease in average colony size of the test fungus under laboratory conditions.

Table 7: ANOVA for colony size at different oil concentrations

Source of Variation	Sum of square	Degree of freedom	Mean square Ratio	Variance
Total	l4421.2	13	-	-
Treatment	4346	6	724.33	98.41**
Plants	31.03	1	31.03	4.21ns
Residual mean square	44.17	6	7.36	

** = Significant at 5% level, ns = Not significant at 5% level.

Conclusion

The overall study can be concluded as

1) Essential oil from *Eucalyptus citriodora* and *Cymbopogon citratus*has antifungal properties. Increase in concentration of both the oils there is decrease in colony size of the test fungus in laboratory conditions which indicates fungicidal characteristics of the used essential oils. Hence, these essential oils might be used as bio fungicides.

2) From comparative analysis of both the oils, it can be concluded that the *Cymbopogon* oil is more effective than the *Eucalyptus* oil for inhibiting the mycelial growth of *Fusarium moniliforme*.

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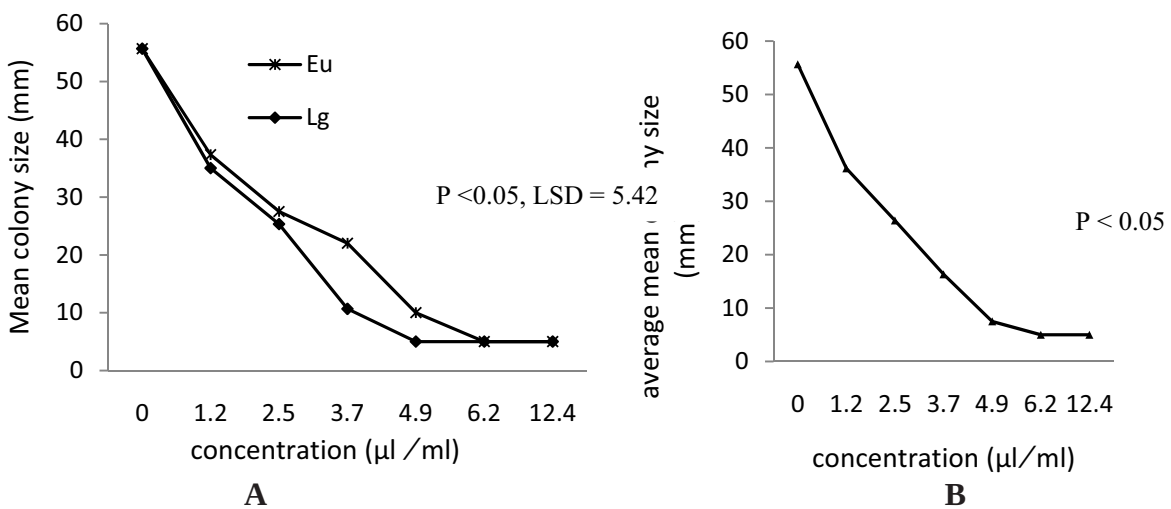

Figure 3: Effect of different concentration of two different oils on the colony size of test fungus (A) and General effect of essential oils on the colony size of the test fungus (B).

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