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ANTIFUNGAL ACTIVITY OF SIX PLANT ESSENTIAL OILS AGAINST PHYTOPATHOGENIC FUNGI *RHIZOPUS ORYZAE*

Monica Verma and Satyawati Sharma

Center for Rural Development and Technology, Indian Institute of Technology Delhi
Hauz Khas, New Delhi - 110 016, India.

Abstract

Members of *Rhizopus* belong to a large group of fungi called Zygomycetes, true fungi with well-developed mycelium with chitin in their cell walls. These fungi are terrestrial and saprophytes or weak pathogens causing soft rots and molds. They grow in soil and on fruits, other foods, and decaying organic materials. *Rhizopus* rot, caused by *Rhizopus nigricans,* can be very destructive to harvested fruit (Peaches, nectarines, sweet cherries, strawberries, and plums). It most commonly affects fruit in storage, during transit, and at the marketplace. However, Rhizopus rot may occur on all decaying vegetation. A few species of *Rhizopus* are known to cause disease in humans as well. *Rhizopus oryzae* is the principal cause of zygomycosis, which occurs primarily in patients suffering from diabetic ketoacidosis (rhinocerebral disease), malnutrition, severe burns, or who are immunocompromised. Chemical fungicides are the most popular method of plant disease control. However, these cause deleterious effects on humans and the environment. Alternative source of fungicides are being searched throughout the world. The present study shows the inhibitory effect of six essential oils (clove, lemongrass, mentha, eucalyptus, orange and turmeric oil) against *R. oryzae*. Fungal growth inhibition bioassay was conducted to test the efficacy of essential oils. Growth inhibition studies suggested that clove oil, lemongrass, and mentha oil were found to be effective against the fungus.

Key words : Antifungal activity, essential oils, pathogenic fungi, *Rhizopus oryzae*.

Introduction

Members of *Rhizopus* belong to a large group of fungi called Zygomycetes, true fungi with well-developed mycelium with chitin in their cell walls. These fungi are terrestrial and saprophytes or weak pathogens causing soft rots and molds. They grow in soil and on fruits, other foods, and decaying organic materials. *Rhizopus* rot, caused by *Rhizopus nigricans* can be very destructive to harvested fruit (Peaches, nectarines, sweet cherries, strawberries, and plums). It most commonly affects fruit in storage, during transit, and at the marketplace. However, Rhizopus rot may occur on all decaying vegetation. A few species of *Rhizopus* are known to cause disease in humans as well. *Rhizopus oryzae* is the principal cause of zygomycosis, which occurs primarily in patients suffering from diabetic ketoacidosis (rhinocerebral disease), malnutrition, severe burns, or who are immunocompromised. Various chemical fungicides are used for the control of disease caused by *R. oryzae*. However, the alternative methods of control are to be searched due to the harmful effects of chemical fungicides, resistance in fungal pathogens, and high production cost of new chemicals.

Natural products derived from plants have been studied by researchers throughout the world to control plant disease. They have many advantages like less environmental effects and low mammalian toxicity. Plant extracts of many higher plants have been reported to exhibit antifungal and insecticidal properties. Plant essential oils are also known to have biopesticidal properties. The specific aroma and flavour prevailing in herbs, spices and perfumes is due to the presence of essential oils. These are volatile aromatic substance found in different parts of the plants such as flowers, buds, seeds, leaves, twigs, bark, woods, fruits and roots. The components responsible for the biological activity and fragrance of essential oils are mono and sesquiterpenes, carbohydrates, phenols, alcohols, ethers, aldehydes and ketones which accounts. Essential oils are having antimicrobial activities. Essential oils have antifungal,

antibacterial, antiviral, insecticidal and antioxidant properties with no adverse effects on humans and animals (Burt, 2004; Sokmen *et al*., 1999). Phenolic compounds present in essential oils have antimicrobial properties (Sumonrat *et al*., 2008).

The essential oil obtained from *Citrus sinensis* fruit peel is called orange oil. Themajor bioactive component present in orange oil is limonene (84.2%) (Sharma and Tripathi, 2006). It has wide-ranging applications in confectionary, toiletry and perfumery industry and are known to exhibit antimicrobial properties such as antifungal, antiviral, antibacterial and antiparasite (Soni and Soni, 2014; Sharma and Tripathi, 2006; Rehman *et al*., 2007).

Curcuma longa (Turmeric) oil is obtained from the rhizome of *C. longa*. The presence of volatile essential oil in the rhizome imparts the characteristic aromatic taste and smell. Eucalyptol (11.2%), α-turmerone (11.1%), β-caryophyllene (9.8%), ar-turmerone (7.3%) and β-sesquiphellandrene (7.1%) are the major constituents of turmeric oil (Raina *et al*., 2002).

The volatile oil extracted from fresh leaves of *Cymbopogon citratus* (Lemongrass) is widely used in cosmetics and perfumes industries (Ferreira and Fonteles, 1989). It has antibacterial and antifungal properties (Inouye *et al*., 2006; Bansod and Rai, 2008; Revathi *et al*., 2012; Yousef, 2013), besides analgesic and anti-inflammatory properties (Negrelle and Gomes, 2007). It constitutes monoterpenes and citral (65-85%) as the major component and small amounts of geraniol, geranylacetate and monoterpene olefins, such as myrcene (Ferreira and Fonteles, 1989).

Eucalyptus globulus leaves contain eucalyptus oil. The oil contains various bioactive components such as cineol (80%), p-cymene, alpha-pinene, limonene, geraniol and camphene. Eucalyptus oil and its constituents have been used for their fungicidal (Ramezani, 2006), herbicidal (Setia *et al*., 2007), insecticidal (Rudin, 2005) properties.

Mentha piperita plant yields mentha oil from all its parts. Mentha oil is commonly used as a flavouring agent in pharmaceuticals, mouthwash, chewing gum, toothpaste and cigarettes. It has also been used in Eastern and Western traditional medicine as an antispasmodic and antiseptic in the cure of cancers, indigestion, nausea, cramps, colds, sore throat and toothaches (Tandan *et al*., 2013). The major compound of mentha oil is menthol (53.28%) (Saharkhiz, 2012). It showed antifungal activities against *Pseudomonas solanacerum*, *Aspergillus niger*, *Alternaria alternata* and *Fusarium chlamydosporum*, respectively (Barrera-Necha *et al*., 2008; Aqil *et al*., 2001; Lirio *et al*., 1998).

Syzygium aromaticum (Clove) essential oil obtained from the dried flower buds of *Syzygium aromaticum* plant. It comprises phenylpropanoids, such as eugenol (76.8%), β-caryophyllene (17.4%) and α-humulene (2.1%) (Chaieb *et al*., 2007). Eugenol (4-allyl-2-methoxyphenol) is the main bioactive component having strong insecticidal, antioxidant and antifungal activity (Chami *et al*., 2005; Gulcin *et al*., 2004 and Park *et al*., 2000).

Keeping these facts in focus the objective of this study was to examine the antifungal activity of six plant essential oils from *Cymbopogon citratus* (Lemon grass oil), *Citrus sinensis* (orange oil), *Curcuma longa* (turmeric oil), *Eucalyptus globulus* (eucalyptus oil), *Mentha piperata* (mentha oil) and *Syzygium aromaticum* (clove oil) against phytopathogenic fungi *Rhizopus oryzae*.

Materials and Methods

Fungal strain

Rhizopus oryzae was procured from IARI, New Delhi. The fungus was cultured and sub-cultured using potato dextrose agar medium and kept in refrigerator at 4°C for further testing.

Essential oils

Plant essential oils from *Cymbopogon citratus* (Lemon grass oil), *Citrus sinensis* (orange oil), *Mentha piperata* (mentha oil), *Curcuma longa* (turmeric oil), *Eucalyptus globulus* (eucalyptus oil) and *Syzygium aromaticum* (clove oil) were procured from local market.

Fungal growth inhibition test/poisoned food technique

To determine the effect of essential oils on growth of fungus, different concentrations of essential oils diluted with acetone in 1:1 ratio were added into Potato dextrose agar media at 0.1, 0.25, 0.5, 0.75, 1, 2.5, 5 % concentration. Treated media (20 ml) was then poured into the petri plate and allowed to solidify. Mycelial plugs (6 mm in diameter) of pure culture of *R. oryzae* were incubated in the center of each PDA plate (9 cm diameter). All the experimental transfers were performed aseptically in laminar air flow. These plates inoculated with fungus were incubated in the dark at 28°C and 70% RH for 7-10 days. Mycelial growth was measured every day until control plates were completely colonized with mycelium. Plates with only media and no oil were used as control. A solvent control was also set up with media and solvent. The experiments were done in triplicates.

Statistical analysis

All statistical analyses were carried out using SPSS 17.0 (SPSS Inc., Chicago, IL). Analysis of variance (ANOVA) was performed on all experimental data and means were compared using Duncan's multirange test. The significance level was $p<0.05$.

Results and Discussion

Antifungal activity of six plant essential oils against phytopathogenic fungi *R. oryzae*

The antifungal activity of different essential oils was studied using growth inhibition assay or poisoned food technique. Essential oils were added in different concentration 0.1, 0.25, 0.5, 0.75, 1, 2.5 and 5% in the media. The results of the antifungal tests showed that the essential oil treated media inhibited the fungal mycelial growth at varying levels. Effect of orange, lemongrass, turmeric, eucalyptus, mentha, and clove oil is shown in Table 1, 2, 3, 4, 5 and 6, respectively.

Effect of orange oil on *R. oryzae*

Different concentrations of orange oil were tested against the fungal growth. It was found that orange oil was not much effective in inhibiting fungal growth. All the concentrations allowed the growth of fungi except the higher concentrations (2.5 and 5%). Controls allowed good growth of fungal mycelium (table 1).

Effect of lemongrass oil on *R. oryzae*

Lemon grass oil was found to be most effective in controlling fungal growth. All the concentrations were able to control the growth of *R. oryzae*. In comparison control showed growth (table 2).

Effect of turmeric oil on *R. oryzae*

Turmeric oil was also not effective like orange oil. However, higher concentrations (2.5 and 5%) effectively restricted the fungal growth. Controls showed normal growth (table 3).

Table 4 : Effect of Eucalyptus oil on the growth of *R. oryzae*.

Eucalyptus oil conc. in media (%)	Colony diameter (cm)						
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
0.1	2.13±0.15c	3.07±0.12c	3.67±0.15c	4.23±0.25b	6.33±0.42b	8.2±0.26 b	9±0 b
0.25	1.97±0.21c	2.73±0.21b	3.23±0.21b	4.1±0.1 b	6.3±0.1 b	8.1±0.1b	9±0 b
0.5	1.93±0.21c	2.5±0.1 b	3.13±0.06b	4±0 b	6.2±0.1 b	7.97±0.06b	9±0 b
0.75	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a
1	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a
2.5	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a
5	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a
Control	1.73±0.11c	2.67±0.15b	4.7±0.10 d	6.03±0.32d	7.03±0.15d	8.33±0.31c	9±0 b
Solvent Control	1.33±0.12b	2.57±0.15b	3.43±0.12b	5.4±0.2 c	6.23±0.25c	8.27±0.31c	8.67±0.58b

Mean± Standard Deviation (SD) on three determinations, Values (means of 3 replicates) in each column not sharing a common letter differ significantly ($p<0.05$) from each other (Duncan's multirange test).

Table 5 : Effect of Mentha oil on the growth of *R. oryzae*.

Mentha oil conc. (%) in media	Colony diameter (cm)						
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
0.1	1±0 b	1.2±0.1b	3.17±0.15b	4.33±0.23b	6.23±0.15b	7.13±0.15b	9.00±0 b
0.25	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a
0.5	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a
0.75	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a
1	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a
2.5	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a
5	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a
Control	1.8±0.44 c	2.53±0.38c	4.13±0.12d	6±0.2 c	7.03±0.15d	8.17±0.21 c	9.00±0 b
Solvent control	1.7±0.26 c	2.47±0.12c	3.73±0.25c	5.6±0.53 c	6.47±0.5 c	8.37±0.35 c	8.67±0.35b

Mean± Standard Deviation (SD) on three determinations, Values (means of 3 replicates) in each column not sharing a common letter differ significantly ($p<0.05$) from each other (Duncan's multirange test).

Table 6 : Effect of Clove oil on the growth of *R. oryzae*.

Clove oil conc. (%) in media	Colony diameter (cm)						
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
0.1	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a
0.25	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a
0.5	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a
0.75	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a
1	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a
2.5	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a
5	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a
Control	1.4±0.1b	2.4±0.2b	3.47±0.25b	5.93±0.35b	6.93±0.45b	8.9±0.2b	9±0 b
Solvent control	1.53±0.15b	2.5±0.26b	3.6±0.17b	5.73±0.49b	6.87±0.15b	8.8±0.35b	8.87±0.23b

Mean± Standard Deviation (SD) on three determinations, Values (means of 3 replicates) in each column not sharing a common letter differ significantly ($p<0.05$) from each other (Duncan's multirange test).

Table 1 : Effect of Orange oil on the growth of *R. oryzae.*

Orange oil conc. (%) in media	Colony diameter (cm)						
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
0.1	1.7±0.1d	2.5±0.1c	2.87±0.12d	3.17±0.15d	4.10±0.1d	4.5±0.26 d	4.87±0.23e
0.25	1.47±0.15bc	1.6±0.1b	1.8±0.26 c	2.33±0.31c	2.83±0.15c	3.2±0.2bc	4.1±0.1 d
0.5	1.3±0.1b	1.1±0.1b	1.6±0.1 c	2.1±0.17b	2.43±0.06b	3.10±0.1 b	3.67±0.06c
0.75	1.1±0.2 b	1.3±0.2b	1.47±0.12b	1.77±0.15 b	2.3±0.1 b	2.7±0.1 b	3.3±0.17b
1	0.83±0.29b	1±0 b	1.17±0.15b	1.6±0.1b	2.1±0.1 b	2.6±0.2 b	3.2±0.3 b
2.5	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a
5	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a
Control	1.67±0.12d	2.4±0.3c	3.9±0.36e	5.57±0.49 e	6.47±0.45 e	7.97±0.15e	9.00±0 f
Solvent control	1.37±0.15b	2.43±0.25c	3.4±0.1e	5±0.2 e	6.33±0.29 e	8.27±0.46e	9.00±0 f

Mean± Standard Deviation (SD) on three determinations, Values (means of 3 replicates) in each column not sharing a common letter differ significantly ($p<0.05$) from each other (Duncan's multirange test).

Effect of eucalyptus oil on *R. oryzae*

The growth of *R. oryzae* was not restricted completely with Eucalyptus oil. The concentrations 0.1 – 0.5% not able to inhibit the growth of fungal mycelium. Fungi grow in control and solvent control plates (table 4).

Effect of mentha oil on *R. oryzae*

Mentha oil also showed good control. All the concentrations controlled growth of mycelia except the lowest concentration of oil (0.1%), which allowed the fungus growth. (Table 5). Control showed excellent growth.

Effect of clove oil on *R. oryzae*

Clove oil was found to be very potent in inhibiting the growth of *R. oryzae*. All the concentration from lower to higher (0.1-5%) showed excellent control over the fungus (table 6). All the concentrations ranging from 0.1-5% inhibited the fungal growth. The control and solvent added plated showed normal growth.

Six essential oils (orange, lemongrass, turmeric, eucalyptus, mentha, and clove oil) were tested against *R. oryzae*. The essential oils showed varied results. Clove oil and lemon grass oil at all concentrations were effective. The active components, eugenol in clove oil and citral in lemon grass oil might be responsible for the antifungal activity. Eucalyptus oil and mentha oil controlled *R. oryzae* to some extent. Cineol in eucalyptus oil and menthol in mentha oil may be responsible for their bioactivity. Orange oil and turmeric oil were not considered to be effective. Only the higher concentrations controlled the fungus and the lower ones allowed the growth. Overall, essential oils were proved to be efficacious in restricting the growth of *R. oryzae* fungal mycelia. The order of efficacy of various essential oil is as follows :

Clove oil = Lemongrass oil › Mentha oil › Eucalyptus oil › Orange oil = Turmeric oil.

Table 2 : Effect of Lemon grass oil on the growth of *R. oryzae*.

Lemon grass oil conc. (%) in media	Colony diameter (cm)						
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
0.1	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a
0.25	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a
0.5	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a
0.75	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a
1	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a
2.5	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a
5	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0a	0±0a
Control	1.63±0.25b	2.17±0.21b	3.4±0.36b	6.03±0.15b	6.63±0.32b	8.32±0.32b	9.00±0b
Solvent control	1.5±0.1b	2.5±0.17c	3.53±0.32b	5.5±0.36b	6.53±0.47b	8.5±0.47b	8.8±0.2b

Mean± Standard Deviation (SD) on three determinations, Values (means of 3 replicates) in each column not sharing a common letter differ significantly ($p<0.05$) from each other (Duncan's multirange test).

Table 3 : Effect of Turmeric oil on the growth of *R. oryzae*.

Turmeric oil conc. (%) in media	Colony diameter (cm)						
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
0.1	1.77±0.6c	2.67±0.15d	2.97±0.15e	3.5±0.2 d	4.57±0.21d	4.7±0.36 e	5.5±0.5 e
0.25	1.6±0.1 c	1.73±0.15c	2.5±0.1 d	2.67±0.06c	3.1±0.36 c	3.93±0.12d	4.5±0. 2 d
0.5	1.47±0.06c	1.57±0.15c	1.87±0.32c	2.33±0.15b	2.6±0.2 b	3.23±0.12c	3.67±0.38c
0.75	1.23±0.15b	1.4±0.2 b	1.53±0.15b	2.2±0.1 b	2.47±0.12b	2.7±0.1b	3.3±0.1 c
1	1±0 b	1.2±0.2 b	1.33±0.06b	2±0.2 b	2.3±0.2 b	2.6±0.1 b	2.8±0.3 b
2.5	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a
5	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a
Control	1.6±0.1 c	2.27±0.21d	3.5±0.3 f	5.2±0.2 e	7.2±0.2 e	8.37±0.32f	9±0 f
Solvent Control	1.63±0.15c	2.23±0.2d	3.4±0.1 f	5.1±0.1 e	6.07±0.12 e	8.23±0.32f	8.87±0.15f

Mean± Standard Deviation (SD) on three determinations, Values (means of 3 replicates) in each column not sharing a common letter differ significantly ($p<0.05$) from each other (Duncan's multirange test)

Conclusion

In this study, six plant essential oils were investigated against phytopathogenic fungus, *R. oryzae*. Growth inhibition studies indicated that clove oil and lemongrass were found to be most potent against the fungus. They completely inhibited the growth of fungus in all the concentrations tested. Mentha and eucalyptus oil were appreciably effective and turmeric and orange oil were not effective. Hence, it is suggested that clove oil and lemongrass oil may be used as natural antifungal agents against *R. oryzae*. Lower concentrations may also be tested to obtain the minimum inhibitory concentrations. These may be further tested in field conditions and formulated to be used as an environmentally safe alternative to chemical fungicides.

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