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# Antimicrobial activity of essential oils against seed borne fungi of rice (*Oryza sativa* L.)

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**Abstract**

Rice (*Oryzae sativa* L.) is one of the most widely cultivated and major food crops of the world. However, despite the use to which it is put, its production is affected by the attack of fungal pathogens. Some of these fungal pathogens move into the field through seeds, ie., they are seed-borne. In the present study, it was observed that *Fusarium moniliforme* and *Helminthosporium oryzae* were present in the seeds of rice, variety ADT 45 and White Ponni. The per cent discolouration varied from 16 to 19. The highest percentage of discolouration (19 %) was found in rice variety ADT 45, followed by White ponni (16 %). A total of two fungal genera were found to be associated with seeds of two different varieties of rice. Among them, the most predominant one was *H. oryzae*, followed by *F. moniliforme.* Six essential oils were tested against two rice seed borne pathogens. The results revealed that three essential oils *viz*., *Cymbopogan citratus, Cymbopogan martini* and *Pelargonium graveolens* were found to be more effective and inhibited mycelial growth of the pathogen with least concentration of 0.1% MIC, while other oils such *Cymbopogan nardus Eucalyptus globulus* and *Ocimum sanctum* were not effective against the pathogens at these concentration. Three essential oils also increased the germination capacity of treated seeds with 85.6% against control (63.0%).

**Key words**

Antifungal activity, Essential oils, *Fusarium moniliforme, Helminthosporium oryzae,* Rice seed

## Introduction

Rice (*Oryza sativa* L.) is the most important cereal crop and one of the major source of calories for large percentage of world population. It covers about 114 countries throughout the world, and more than 50 countries have production of 100,000 t (Reddy *et al.*, 2004). In India more than half of the population depends on rice for food. India is the major rice growing countries with an area of 44 m ha and production of 100 million tones. It is cultivated in almost all the states of India, contributing about 42% to the country's food grain production and provides livelihood for about 70% of the population (Prasad *et al*., 2012). About 90% of rice is being produced in Asian countries, particularly China and India being the major producer. The other major producing countries are Indonesia, Bangladesh, Vietnam, Thailand, Myanmar, Philippines, Brazil and Japan (IRRI, 2008).

Fungi are a major cause of reduction in the quality of rice due to high moisture and temperature conditions before its harvest. They produce different types of symptoms such as seed abortion, seed rot, seed necrosis, reduction or elimination of seed germination resulting in the development of disease at later stages of plant growth (Khanzada *et al*., 2002). Apart from being seed borne pathogens, fungi may grow on storage products. Consumption of infected seeds by man and domestic animals results in the production of toxins (Uma and Wesely, 2013). Rice seeds are known to harbor a wide range of both fungi and bacteria (Neergaard, 1977). During harvesting, continuous and heavy rainfall may wet

the crop and make panicles more prone to invasion by fungal pathogen (Reddy *et al.*, 2004). Kakoly *et al* . (2014) reported yield loss of approximately 26 to 46 % due to infected grains.

Chemical and biological methods have been used to control wide range of seed borne diseases in rice. Chemical methods, such as foliar application or fumigation, are harmful to the environment. Also, dramatic increase in resistance of the pathogens to chemicals, and toxicity and accumulation of chemicals in the environment have prompted interest in alternative forms of disease control (Cook, 1993). Hence, the development of natural products as alternatives to synthetic fungicides is required. The recent efforts have focused on developing environmentally safe, long lasting and effective essential oils for controlling plant diseases.

Biologically, active essential oils represent a rich source of an alternative and perhaps, environmental more acceptable disease compounds. With a broad range of natural fungicidal plant volatiles, numerous opportunities exist to explore their usefulness in controlling plant disease. With this background the following objectives were formulated in the present study to isolate and identify major seed borne fungi of paddy (var- ADT 45 and White ponni); to assess cultural characteristics of isolated pathogen; to screen antifungal activity of selected essential oils against seed borne fungi of rice; and to test the selected essential oils as seed treatment against pathogen.

## Materials and Methods

**Collection of seed samples :** Two rice varieties (ADT 45 and White ponni) were collected from the Experimental Farm, Department of Agronomy, Faculty of Agriculture, Annamalai University, Tamil Nadu, India. Seeds were collected in sterilized polythene bags and stored at 4-5°C until further use. Randomly 100 seeds were picked from 400 seeds of each of variety. These seeds were further divided into two categories such as normal seeds and discoloured seeds. Based on these visual observations per cent discolouration was calculated.

**Isolation and identification of pathogen associated with rice seeds (Blotter paper method) :** Standard blotter method was used to determine the occurrence of seed borne mycoflora on rice seeds (Mathur and Kongsdal, 2003). From each variety, 400 seeds were collected and 100 seeds were picked out randomly and were subjected to isolation. A set of three plates (100 seeds) were considered as replicates. Petri plates containing 25 seeds were considered as sub-replicates. Three pieces of filter paper were soaked in sterilized water and placed at the bottom of 9 cm well labelled Petri dishes. Seeds were placed and spaced in each Petri plate using a pair of forceps, making sure that seeds were placed equidistantly with 15 seeds on the outer ring, 9 seeds at inner ring and 1 seed in the middle. The lids of each Petri plates were held in place with gummy cello tape. The Petri plates containing seeds were incubated at 28 ±2° C for 7 days under alternating cycles of 12 hrs near UV light and 12 hrs of darkness.

After 7-day of incubation period, individual rice seeds were examined under Stereo Binocular microscope (CX 21i) in order to record the incidence of different seed borne fungi. Fungi associated with rice seed samples were recorded and expressed in percentage individually. With flamed-sterilized transfer needle, fungal growth on grains were aseptically mounted in lacto phenol blue on slides and examined under stereo-binocular microscope for fungal diagnostic characteristics. For proper identification of fungi, semi permanent slides were prepared from the fungal colony and observed under compound microscope. Pure cultures of isolated fungi were obtained through transfer on Potato Dextrose Agar medium (Ainsworth, 1961). Fungi were identified on the basis of their typical structure and basic characters as suggested by Booth (1971) and Watanabe (2002).

**Collection of essential oil :** Locally available six essential oils *viz.*, Citronella oil (*Cymbopogon nardus*-Graminae), *Eucalyptus* oil (*Eucalyptus globules*-Myrtaceae), Geranium oil (*Pelargonium graveolens*-Geraniaceae), Lemongrass oil (*Cymbopogon citratus*-Graminae), Palmarosa oil (*Cymbopogon martini*- Graminae) and Tulsi oil (*Ocimum sanctum*-Labiate) were purchased from Citro Essential Oils Distillery Industry, Erode, Tamil Nadu (India). These essential oils were selected on local availability and previous knowledge of their antifungal activities (Ibiam *et al.,* 2008; Nguefack *et al*., 2008).

**Antifungal activity of essential oils against major seed borne fungi of rice :** Antifungal activities of six essential oils were assessed against four major seed borne fungi by radial growth assay following poisoned food technique (Nehal and EI-Mougy, 2009). Plant oils were tested in the concentration range of 0.02 to 0.1% (v/v). To 50 ml sterilized PDA, different concentrations of plant oils were mixed separately and dispensed to sterile Petri plates. All the plates were gently rotated for even dispersal of oil. Plates with 0.1% thiram were included as standard check and plates without oil served as control. Eight mm discs of test fungi taken from the advancing edge of test pathogens were placed in oil-containing PDA plates and incubated at 28±2°C. The incubation period differed depending upon the pathogen. Four replicates for each treatment were maintained. At the end of incubation period, colony diameter (mm) of test pathogens was measured to determine the Minimum Inhibitory Concentration (MIC) of respective oil.

**Efficacy of seed treatment with selected essential oils against seed borne fungi**

**Seed treatment :** For each rice cultivar, seeds were collected from the experimental farm, and seed lots were divided into required number of sub-lots (based on number of treatments) of 50 g each. Eight sub-lots were treated with a concentration range of selected essential oil (based on *in vitro* assay) along with chemical control and untreated control (sterile water), respectively. For seed treatment, the soaking method of Gangopadhyay and Kapoor (1977) was followed. Efficacy of essential oils against seed borne fungi was evaluated, based on the standard blotter method (Nene and Thapliyal, 1993) and seed germination test (ISTA, 2003). The treatment schedule followed was: $T_1$-*Cymbopogan citratus* @0.1%; $T_2$-*Cymbopogan martinii* @0.1%; $T_3$-*Pelargonium graveolens* @0.1%; $T_4$-Thiram @2g/kg of seed and $T_5$-Untreated control.

**Roll towel method :** For each treatment, 200 seeds (ADT 45 and white ponni) were tested in three replicates of 50 seeds each, using the between paper (BP) method including fungicide treated and untreated (sterile water) control. After 7 and 14 days of incubation, the seedlings were evaluated for normal, abnormal seedlings and fresh ungerminated seeds according to the International Rules for Seed Testing (2003) and expressed in percentage.

$$MPG = N1\text{-}N2/N1 \times 100$$

where, MPG is the mean percentage germination; N1 is the number of treated seeds placed; N2 is the number of un-germinated seeds.

**Statistical analysis :** All the experiments were of completely randomized design (CRD) and repeated twice. Data were subjected to analyses of variance and treatment means were compared by Duncan's multiple range test ($P<0.05$). The IRRISTAT package version 92-1, developed by the International Rice Research Institute Biometrics Unit, Philippines, was used for analysis (Gomez and Gomez, 1984).

## Results and Discussion

Incidence of grain discolouration in two different rice varieties under natural field conditions is presented in Table 1 (Plate 1). The per cent discolouration varied from 16 to 19. Highest percentage of discolouration (19 %) was found in rice variety white ponni, followed by ADT 45 (16 %).

Discolouration of grains is an everlasting problem in rice growing countries, as it is mainly caused by fungi with congenial environment. Due to this, there is a loss in weight of grains, nutritive quality and hazardous to consumer, perhaps due to the presence aflatoxins. The range of discolouration may vary from variety to variety and location to location. This variation may be due to variation in farmer knowledge regarding seed production and processing (Ahmed *et al*., 2013). Panicle submergence with lodging is the reason for seed discolouration (Mettananda *et al*., 2001).

The results of the present study is in agreement with the findings of Uma and Wesely (2013) who reported per cent discoloutration of rice seeds varying from 28 to 46. Seed discolouration percentage in different rice varieties has also been reported by Raj *et al. (*2007); Somda *et al*. (2008) and Naqvi *et al*. (2013).

A total of two fungal genera were found to be associated with seeds of two different rice varieties (Table 2); (Fig. 2a, 2b and 3). The associated fungi were *F. moniliforme* and *H. oryzae* (Fig. 3). Among them, the most predominant one was *H. oryzae* which was associated with 15.3 % (white ponni) and 13.0% (ADT 45) of seed samples, followed by *F. moniliforme* (8.3 % and 7.6 %). Among these, *H. oryzae* was the most frequently isolated seed-borne fungus irrespective of the source of two different rice varieties tested in the present study.

**Table 1 :** Occurrence of discoloration in rice varieties (White ponni and ADT 45)

| Locality | District | Variety | Discoloured grains (%) |
|---|---|---|---|
| Experimental farm, Annamalai University | Cuddalore | White ponni | 16 [b] |
| Experimental farm Annamalai University, | Cuddalore | ADT 45 | 19 [a] |

In a column means followed by a common letter are not significantly different at 5 per cent (P=0.05) level by DMRT

**Table 2 :** Fungi associated with rice varieties

| Variety | Percentage of association | |
|---|---|---|
| | *Fusarium moniliforme* | *Helminthosporium oryzae* |
| White ponni | 8.3 [a] | 13.0 [b] |
| ADT 45 | 7.6 [b] | 15.3 [a] |

In a column means followed by a common letter are not significantly different at 5 per cent (P=0.05) level by DMRT


**Fig. 1 :** Rice varieties of ADT 45 and White ponni

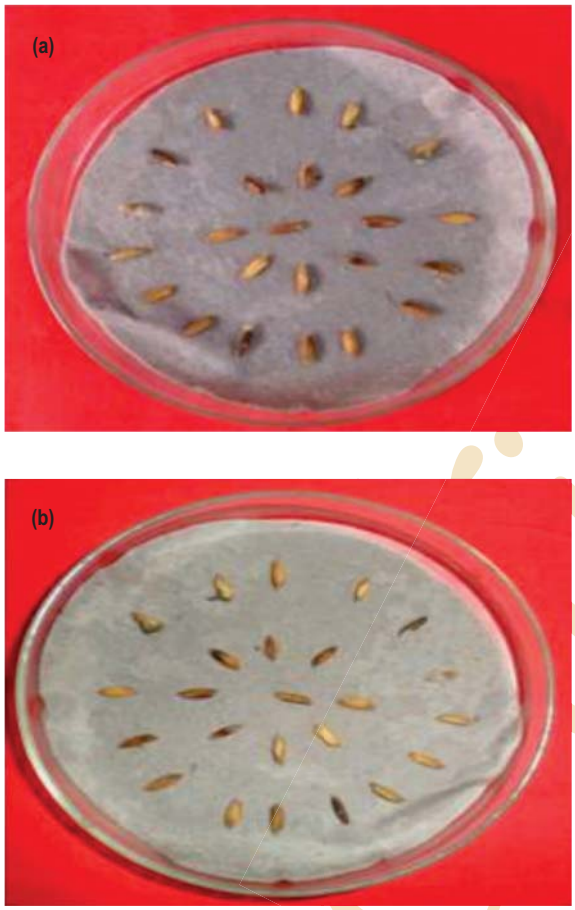


**Fig. 2 :** Isolation of pathogen (Blotter method) (a) ADT (b) White ponni

Gopalakrishnan *et al.* (2010) observed total eight genera of fungi to be associated with rice seeds. Among them, the most predominant one was *H. oryzae* which was associated with 58.59% seed samples, followed by *A. padwickii* (52.96%). Recently, Archana and Prakash (2013) reported a total of 69 rice seed samples comprising of six genera of fungi to be associated with rice seed bags. Among them, the most predominant one was *H. oryzae* which was associated with 82. 08% seed samples, followed by *A. padwickii* (63.36%).

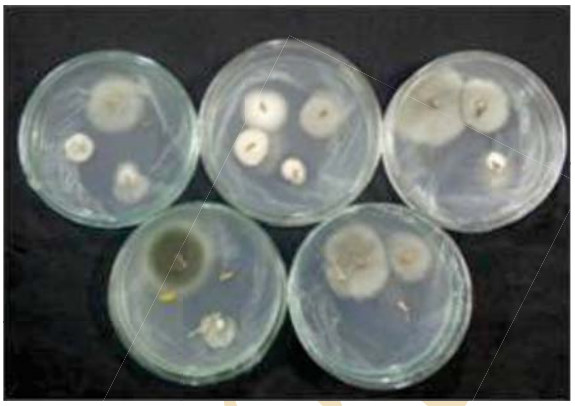

**Fig. 3 :** Pathogen grown on PDA medium

Growth of *F. moniliforme* colony on PDA was fast growing, initially white, becoming lavender tinged, colourless to dark purple in reverse. Hyphae were hyaline and septate. Conidiophores were short, simple or branched. Macro conidia were sparse, simple or branched and slightly sickle shaped to almost straight and septate. Micro conidia were abundant, single celled, oval to club shaped forming long chains (Fig. 4a, b).

Whereas, *H. oryzae* coloy on PDA produced aerial mycelium which was fluffy, cottony, grey olivaceous, with brownish tinge. Conidiophores were single or in small groups, straight to flexuous, sometimes geniculate, pale to mid brown or olivaceous brown, pale towards the apex and septate. Conidia were usually curved, navicular, fusoid or obclavate, occasionally almost cylindrical, pale to mid golden brown and smooth (Fig. 4c, d).

Out of six essential oils tested, three oils showed fungicidal properties against *F. moniliforme* with MIC range of 0.1% (Plate 5). Three essential oils *viz*., *C. citratus*, *C. martinii* and *P. graveolens* was found to be more effective and inhibited growth of pathogen with least concentration of 0.1%, while *C. nardus, E. globules* and *O. sanctum* oils were effective at 0.1% MIC (Table 3) (Fig 5a, b and c). This is in agreement with findings of Somda *et al*. (2008) who reported that the essential oil of *C. citratus* exhibited strong antifungal activity against *F. moniliforme* even at low concentration. Citral oil completely inhibited mycelial growth of *A. alternata, F. moniliforme* and *F. pallidoroseum* in paper disc assay (Kishore *et al.*, 2007), where oil of *O. sanctum* showed highest antifungal activity against *F. solani* (Joseph *et al*., 2008).

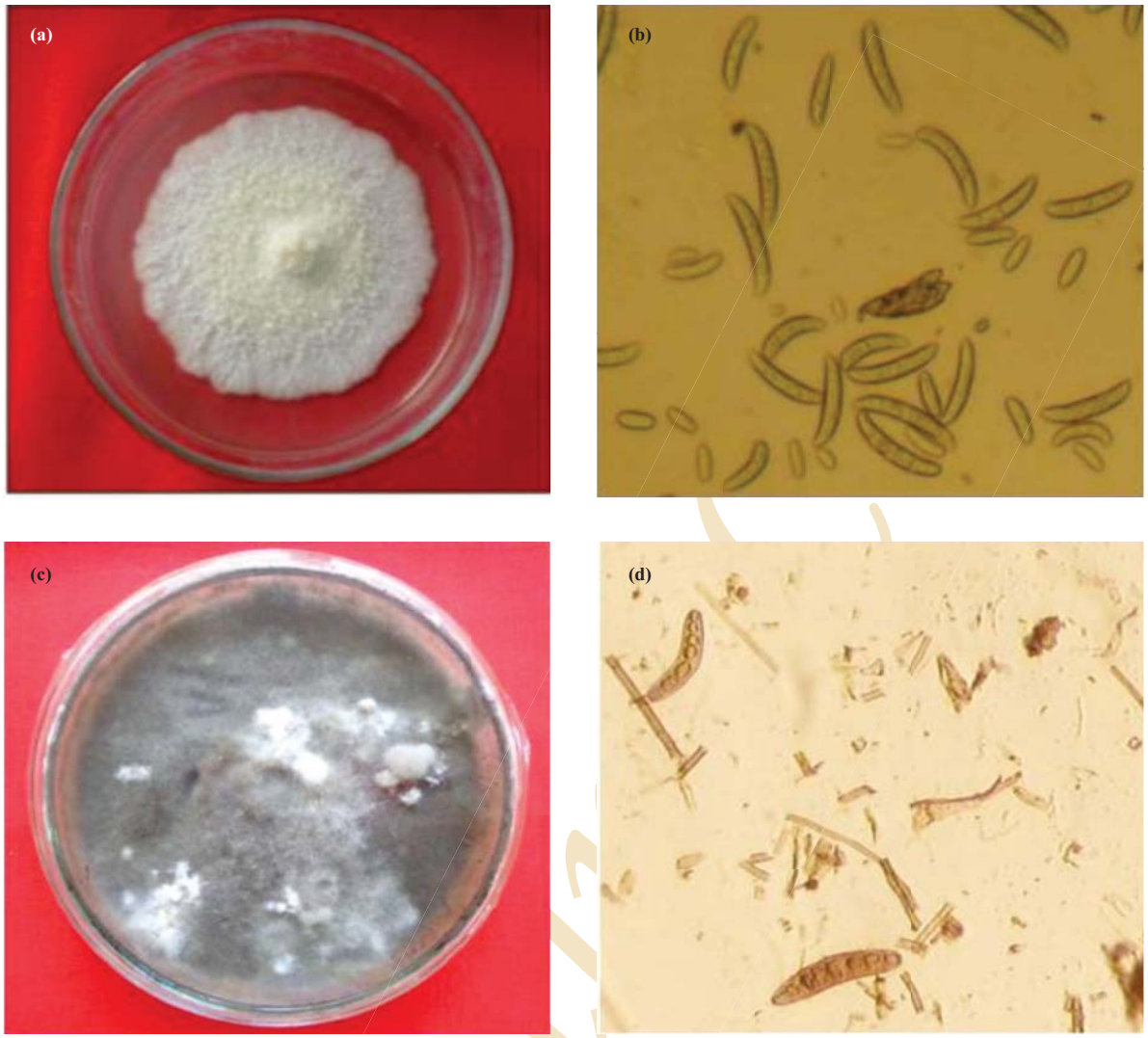


**Fig. 4 :** Axenic culture and conidial characteristics of seed borne fungus from rice seeds (a) Culture of *Fusarium moniliforme;* (b) Conidia; (c) Culture of *Helminthosporium oryzae* and (d) Conidia

**Table 3 :** Inhibition of *Fusarium moniliforme* by essential oils

| Essential oils | Concentration (%) / Radial growth of pathogen (mm) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **0.02** | **0.03** | **0.04** | **0.05** | **0.06** | **0.07** | **0.08** | **0.09** | **0.1** |
| *Cymbopogan citratus* | 42.3 [b] | 37.6 [a] | 30.3 [a] | 24.6 [a] | 19.0 [a] | 12.6 [a] | 7.3 [a] | 1.9 [a] | 0.0 [a] |
| *Cymbopogan martinii* | 46.6 [a] | 39.3 [a] | 31.0 [a] | 26.3 [a] | 19.9 [a] | 13.6 [a] | 8.9 [a] | 2.3 a | 0.0 [a] |
| *Cymbopogan nardus* | 56.0 [c] | 49.3 [b] | 43.6 [b] | 37.3 [c] | 30.9 [c] | 25.9 [c] | 20.9 [c] | 14.3 [c] | 7.9 [c] |
| *Eucalyptus globulus* | 54.6 [c] | 47.3 [b] | 41.3 [b] | 34.9 [b] | 28.6 [b] | 22.3 [b] | 17.6 [b] | 11.3 [b] | 5.9 [b] |
| *Ocimum sanctum* | 62.0 [d] | 56.3 [c] | 49.6 [c] | 42.3 [d] | 35.6 [d] | 28.6 [d] | 21.3 [c] | 16.6 [d] | 10.3 [d] |
| *Pelargonium graveolens* | 43.6 [b] | 37.9 [a] | 32.3 [a] | 26.6 [a] | 20.3 [a] | 13.9 [a] | 8.3 [a] | 3.3 [a] | 0.0 [a] |
| Control | 88.0 [e] | 88.4 [d] | 89.0 [d] | 88.4 [e] | 88.4 [e] | 88.6 [e] | 89.0 [d] | 88.6 [e] | 88.0 [e] |

In a column means followed by a common letter are not significantly different at 5 % level by DMRT; 0.0-Complete inhibition

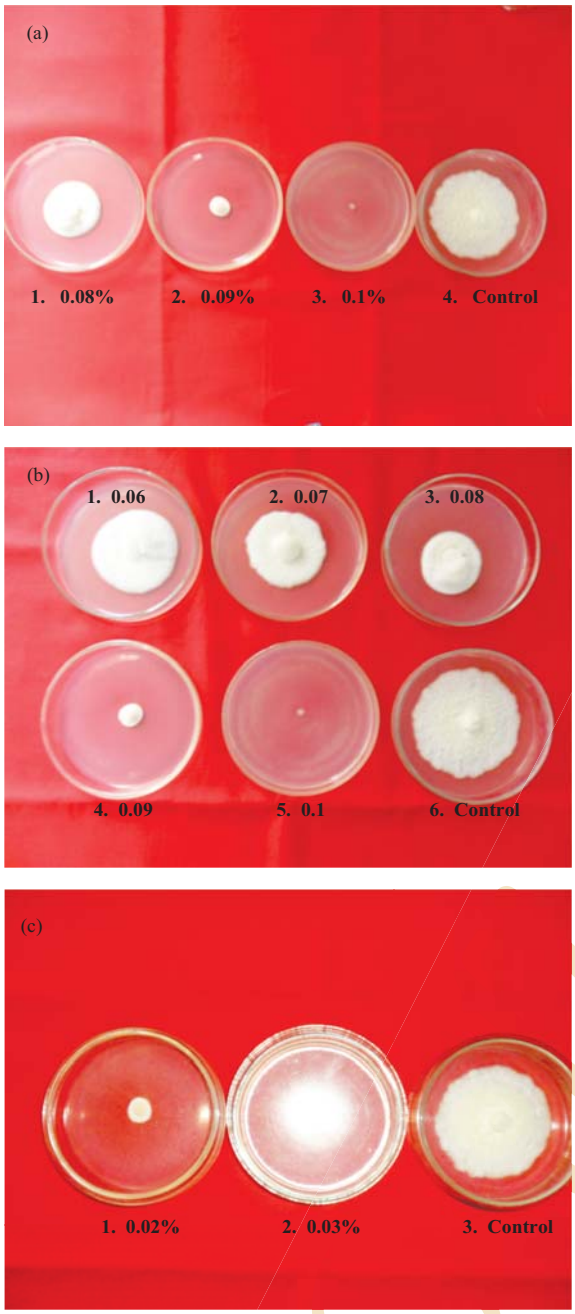


**Fig. 5 :** Minimum inhibitory concentration of essential oils on the growth of *Fusarium moniliforme* (a) Efficacy of Palmarosa oil on the growth of *Fusarium moniliforme* (b) Efficacy of Lemongrass oil on the growth of *Fusarium moniliforme* (c) Efficacy of Geranium oil on the growth of *Fusarium moniliforme*

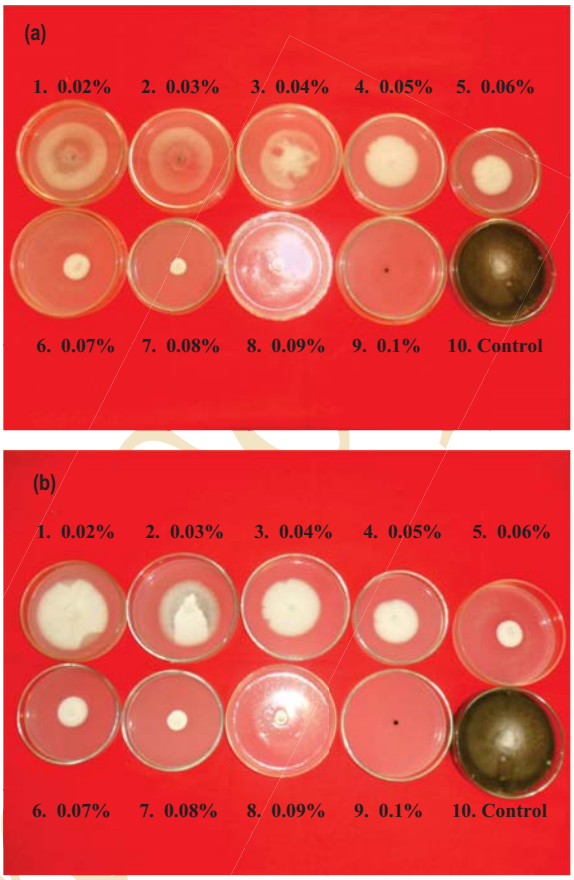


**Plate 6 :** Minimum inhibitory concentration of essential oils on the growth of *Helminthosporium oryzae* (a) Efficacy of Palmarosa oil on the growth of *Helminthosporium oryzae* and (b) Efficacy of Lemongrass oil on the growth of *Helminthosporium oryzae*

Out of six essential oils tested, two showed fungicidal properties against *H. oryzae* with MIC range of 0.1% (Fig. 6). The essential oils of *C. citratus, C. martinii* and *P.graveolens* were found to be more effective and inhibit the growth of pathogen with least concentration of 0.1%, while the other oils were not effective at 0.1% MIC (Table 4) (Fig. 6a, b). These results are in confirmation with the reports of Nguefack *et al*. (2008) and (2013) Essential oils of *C. citratus, O. gratissimum* and *T. vulgaris* were highly effective in inhibiting the mycelial growth of *H. oryzae* and *A. alternata*, the cause of rice seed borne pathogen.

Antifungal compounds present in the essential oil of *P. graveolens* include citronellol, geraniol, citronellyl formate and linalool (Ana *et al*., 2014). α-citral, z-citral, limonene, caryophyllene, cerenl acetate, geraniol and citral are the main components of *C. citratus* oil (Farhang *et al.,* 2013). The antifungal action of palmarosa oil is mainly attributed to its geraniol content. In addition to geraniol, geranyl acetate, linalool and β-carboxy phyllene have also been reported to possess antifungal activity (Anjali *et al*., 2003).

**Table 4 :** Inhibition of *Helminthosporium oryzae* by essential oils

| Essential oils | Concentration (%) / Radial growth of pathogen (mm) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **0.02** | **0.03** | **0.04** | **0.05** | **0.06** | **0.07** | **0.08** | **0.09** | **0.1** |
| *Cymbopogan citratus* | 40.0 [a] | 35.3 [a] | 30.6 [a] | 24.3 [a] | 19.0 [a] | 13.6 [a] | 6.9 [a] | 1.3 [a] | 0.0 [a] |
| *Cymbopogan martinii* | 47.0 [b] | 40.3 [b] | 35.0 [b] | 28.6 [b] | 20.3 [a] | 14.9 [a] | 8.3 [a] | 2.6 [a] | 0.0 [a] |
| *Cymbopogan nardus* | 65.0 [c] | 57.0 [d] | 50.0 [d] | 43.6 [d] | 37.3 [c] | 31.3 [c] | 24.0 [c] | 18.6 [c] | 9.6 [c] |
| *Eucalyptus globulus* | 82.0 [d] | 78.0 [e] | 69.0 [e] | 62.0 [e] | 54.0 [d] | 43.9 [d] | 35.0 [d] | 24.6 [d] | 15.3 [d] |
| *Ocimum sanctum* | 66.0 [c] | 54.0 [c] | 48.0 [c] | 41.0 [c] | 35.0 [b] | 23.0 [b] | 17.6 [b] | 11.9 [b] | 7.6 [b] |
| *Pelargonium graveolens* | 42.0 [a] | 36.3 [a] | 30.9 [a] | 25.6 [a] | 19.6 [a] | 14.0 [a] | 7.6 [a] | 1.9 [a] | 0.0 [a] |
| Control | 88.4 [e] | 88.6 [f] | 89.0 [f] | 88.4 [f] | 88.4 [e] | 88.6 [e] | 89.0 [e] | 88.6 [e] | 89.0 [e] |

In a column means followed by a common letter are not significantly different at5 per cent (P=0.05) level by DMRT; 0.0-Complete inhibition

**Table 5 :** Activity of rice seed germination by essential oils

| Essential oils | White ponni | | ADT 45 | |
|---|---|---|---|---|
| | **Conc. of oils (%)** | **Germination (%)** | **Conc. of oils (%)** | **Germination (%)** |
| $T_1$-*Cymbopogan citratus* | 0.1 | 85.6 [a] | 0.02 | 84.3 [a] |
| $T_2$-*Cymbopogan martinii* | 0.1 | 83.0 [b] | 0.02 | 82.6 [a] |
| $T_3$-*Pelargonium graveolens* | 0.1 | 81.0 [c] | 0.02 | 80.3 [b] |
| $T_4$-Thiram | - | 84.6 [b] | - | 83.6 [a] |
| $T_5$-Untreated control | - | 63.0 [d] | - | 61.9 [c] |

In a column means followed by a common letter are not significantly different at5 per cent (P=0.05) level by DMRT

Among the plant oils tested by Blotter method, *C. citratus* was best for rice seedlings. Seed soaking with *C. citratus* oil, thiram followed by *C. martinii* oil increased seed germination percentage by 85.6% against control which recorded 63.0% (Table 5). Schwinn (1994) stated that any bioagents used as seed treatment purpose should protect both seed and soil-borne pathogens, control deep-seated seed-borne pathogens and stimulate germination and or, enhance growth during the seedling stages.

Germination of rice seedlings raised from seeds treated with essential oils were better compared to the seedlings of untreated seeds. The enhancement of seed germination by natural products might be due to several factors such as fungitoxic actions, leading to killing of pathogens present both internally and externally in seeds. Similarly, Ngufack *et al.* (2008) reported that rice seeds treated with *C. citratus, T. vulgaris* and *O. gratissimum* recorded high germination as compared to Dithane M-45 treated seeds. Seed treatment with *C. citrinus* oil significantly increased the emergence of rice seedling by 16% (Nguefack *et al.,* 2007; Nguefack *et al*., 2013). This is to our knowledge the first report on *C. citratus, C. martinii* and *P. graveolens* oils as germination stimulant of rice seeds.

The present study revealed that among the essential oils tested, *C. citratus, C. martinii* and *P. graveolens* oils were more effective and completely inhibited mycelial growth of pathogen even at least concentration of 0.1%, while other oils were in effective at 0.1% MIC *in vitro*. Seed soaking with these oil increased seed germination percentage as compared to untreated control. In future, these oils will be formulated and can be tested under filed conditions as natural fungicides.

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