1. INTRODUCTION

1.1 General Background

Nepal is an agricultural country and more than 80% people are dependent upon agriculture. Rice is staple food for most of the people in the world and in context of Nepal it is most important cereal crop. Rice is grown over acreage of about 146 million hectors in the tropical and subtropical part of the world (Kochar 1998). Nepal produced merely 2372020 metric tons of paddy in the year 1986\87 which increases to 4455722 metric tons in the year 2003/2004 and the total production in the year 2005/2006 is 4209279 metric tons. The area of paddy cultivation was 1333360 hectare in 1986/1987, 1559436 hectares in 2003/2004 and 1549447 hectares in 2005/2006. Similarly, the annual yield of paddy in 1986/87 was 1779 kg per hectare, in 2003/2004 it was 2857 kg per hectare and in 2005/2006 it was 2717 kg per hectare (Ministry of Agriculture and Cooperatives 2005/2006). Most of the districts in Terai region of Nepal produce the bulk of rice to fulfill the demand of other hilly districts. Morang district has the highest coverage of paddy field with 98070 hectares and Jhapa district is in the second rank with 96800 hectares (Ministry of Agriculture and Cooperatives 2005/2006). The total production of Morang district alone exceeds the rice produced by more than 22 hilly districts put together (Nepal news.com, July 16, 2004). Due to topography and weather, Kathmandu valley and hill districts rely on single rice crop whereas the Terai produces two paddy crops a year.

However agriculture productivity has remained almost stagnant since the last decade and is rather low. Agricultural productivity in Nepal is 25-50 % lower than the world average (Jha and Karmacharya, 2003). At the same time, the nation has to face the challenges of an ever increasing population. Since prospects for increasing agricultural production by expansion of land under cultivation is limited, using improved cultivation practices with proper management of pests are the only means of increasing food production (Amatya *et al*. 1992).

The area, production and yield of paddy in Nepal during last 10 years are given below:

Year	Area (Hectare)	Production (Metric tons)	Yield (Kg/ hectare)
1996/97	1511230	3710650	2455
1997/98	1506340	3640860	2417
1998/99	1514210	3709770	2450
1999/2000	1550990	4030100	2598
2000/2001	1560044	4216465	2703
2001/2002	1516980	4164687	2745
2002/2003	1544660	4132500	2675
2003/2004	1559436	4455722	2857
2004/2005	1541729	4289827	2782
2005/2006	1549447	4209279	2717

Source: Ministry of Agriculture and Co-operatives, 2005/2006.

1.2 Rice plant and its diseases

Rice is a semi aquatic, free tillering annual grass with a cylindrical jointed stem. Rice is primarily a plant of hot moist tropics. It prefers a climate where the average summer temperature does not grow below 77° F. It grows best on damp soils under laid with a semi – impervious subsoil in places where it can be flooded. The decrease in average productivity of rice grain in Nepal may be due to lots of environmental and biological factors. Among the biological factors disease causing pathogens such as fungi, bacteria and viruses are the major ones. Blast disease of Rice caused by *Pyricularia oryzae*, Brown spot disease of Rice caused by *Helminthosporium oryzae*, Foot rot of Rice caused by *Fusarium moniliforme*, False smut of Rice caused by *Ustilaginoidea virens*, Bacterial leaf blight of Rice caused by *Xanthomonas oryzae*, Tungro disease of Rice caused by Rice tungro virus etc. are some of the well-known reported fungal, bacterial and viral diseases of rice from Nepal. Many synthetic fungicides have been used to control the disease caused by fungi, bacteria, nematodes and viruses. Even chemicals are better controlling agents however they have many side effects. In such case applying of biopesticides may be one of an alternative way to get- rid- of from such fungal pathogens.

1.3 Foot rot disease

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 rot or elongation disease is widespread in many rice growing areas in both tropical and temperate regions. They are reported to cause heavy crop loss by damaging the rice plant in seedling stage. Ito and Kimura (1931) reported up to 20% losses in Hokkaido. Pavgi and Singh (1964) stated that losses of 15% occurred in Eastern districts of Uttar Pradesh (India) and Kanjanasoon (1965) found 3 – 14.7% loss in Northern and Central Thiland.

1.3.1 Symptoms

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. The affected seedlings become thin, pale and tall. Such symptoms appear from sixth day after sowing in wet nurseries and continue up to the sixth week. As soon as the symptoms appear the seedling wilt and die within 3 to 6 days. In the seventh or eighth week, it is noted that the older diseased plants have produced fewer tillers than the healthy plants. In sever infection, either the panicle does not develop or a whitish sterile ear is developed. The infected plants may be seen conspicuously above the general level of the crop. Another conspicuous symptom is the development of adventitious roots from the lower nodes of the culm, above the ground level. Such adventitious roots may be partially or completely enclosed by the leaf sheath. If the infected culms are splitted, the brown discoloration of the nodal tissues is seen. The mycelia mats and spores fill up the hollow internodes of infected plants. In later stages the mycelium appears outside in the basal nodal zones, resulting in the formation of a pinkish bloom on the surface. Ultimately the nodes become rotten, and the culm breaks at this point when pulled. The most peculiar symptom of this disease is the elongation of plants followed by death (Pandey, 2003).

1.3.2. Disease cycle

Generally, the disease is seed borne and sometimes may be soil borne. The stubbles and plant debris left in the field after harvest cause infection through the soil. 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.

1.3.3 Control measures

Since the disease is seed borne, various methods of seed treatment have been suggested for the control of this disease. Some of them are as follows:

1. Steeping of grains in 1% formalin for 15 minutes.

2. Use of organomercury compounds such as Agrosan, Certosan, Ceresan, Harvesan at the rate of 2 g / kg seed has been proved very effective.

3. Successful control has been achieved by hot water treatment of the grain at 55°C for half an hour.

4. Use of biological pesticides.

5. Cultivation of resistant varieties.

1.4 Test fungus (*Fusarium moniliforme* sheld.)

The pathogen is world wide in distribution and also parasitizes the other graminaceous hosts such as sorghum, maize and sugarcane. The fungi may grow readily on the number of media, producing luxuriant mycelium. The hyphae are slender, 3-5 µ broad, closely septate and much branched. Each microconidium is 1-2 celled, elliptic to ovate or oval in shape and measures 5-12 x 2-4 µ. The macroconidium is 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 asci measure 75-130 x 9-16 micron. The ascospores are long ellipsoid, one-septa and measure 10-20 x 4-9 micron. Each ascus contains 4 or 6 ascospores (Pandey, 2003).

1.5 Test plant species

Two plants *Eucalyptus citriodora* and *Cymbopogon citratus* were taken in experiments. These plants are easily available aromatic plants of 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. These plant species are rich in essential oils and has been used for many purposes.

1.5.1 *Eucalyptus citriodora* Hook.

(Family- Myrtaceae)

The plant is an evergreen tree 24–40 m high with tall straight trunk 0.6–1.3 m in diameter, and thin, graceful crown of drooping foliage. Bark smooth, gray, peeling off in thin irregular scales or patches and becoming mottled, exposing whitish or faintly bluish inner layer with powdery surfaces appearing dimpled. Twigs slender, slightly flattened, light green, tinged with brown. Leaves alternate, narrowly lance-shaped, 10–20 cm long, 1–2.5 cm wide, apically acuminate, basally acute, entire, glabrous, thin, light green on both surfaces, with many fine parallel straight veins and with vein inside edge. Corymbs terminal and at leaf base 5 to 6 cm long, branched. Flowers many, 3–5 on equal short stalks (umbels) from ovoid buds 8–12 mm long, 5–8 mm wide. Stamens many, thread likes, white, 6 mm long, spreading ca 12 mm across, anthers with long gland. Pistil inferior 3-celled ovary and long, stout style. Capsules few, urn-shaped or ovoid, narrowed into short neck, 10–12 mm long, 8–10 mm wide, brown with scattered raised dots. Seeds few, irregularly ellipsoid, 4–5 mm long, shiny black (Little, 1983). Main components from the plant reported are Citronnellal- 66%, Citronnellol-12%, Citronnellyl acetate-4%, Isopulegol- 3% from 86% oxygenated compound (Fandohan *et al.* 2004).

1.5.2 *Cymbopogon citratus* (DC) Stapf.

(Family- Poaceae)

The plant is a tall tufted perennial with a short rhizome. It is a cultigens which rarely flowers, widely distributed allover the tropics of the hemisphere. The roots are fibrous and leaves are graminaceous with parallel venation. The leaves posses silica lining and is rough in touch. Many glands all over the body especially in leaves and rhizome are present. The plant is lemon scented and have strong aroma.

It is cultivated in Java, Straits Settlements, Ceylon, Burma, Madagascar, Mauritius, West Indies and all parts of South Africa. The grass flourishes in well drained sandy soil. It grows equally well in acid, light colored, micacaeous loam. The essential oil is commercially produced in high scale in Madagascar and Comoro Island. An infusion of the grass sometimes taken as a refreshing beverage. The oil is soluble at 70 % alcohol (Anonymous 1998). Main components from the plant reported are Myrcene- 28%, Neral (Citral B) - 20%, Geranial (Citral A) - 27%, Geraniol- 4% from 61% oxygenated compound (Fandohan *et al.* 2004)

1.6 Objectives

1) To study the effect of essential oils from *Eucalyptus citriodora* and *Cymbopogon citratus* on mycelial growth of test fungus.
2) To study the effect of essential oils from *Eucalyptus citriodora* and *Cymbopogon citratus* against fungal contamination on rice seeds.

1.7 Justification

Rice is an important cereal crop of Nepal since it is the main staple food. On the other hand foot rot of rice caused by *Fusarium moniliforme* has been destructively distributed in rice growing areas. It is one of the dangerous diseases which attack the plant in seedling stage. Control of this disease through the use of different fungicides is a

common method. But fungicides are much hazardous to man and environment. A report from Research council, Board of Agriculture 1987 about pesticides residue on food suggests that fungicides have more of a carcinogenic risk than insecticides and herbicides together. Therefore, plant extracts are less toxic to the plants in comparison to the synthetic fungicides. Hence the essential oils can be use to control the disease caused in plants by fungal pathogens.

1.8 Limitation of the study

Due to time and economic factors the study suffers from following limitation

1. Study was carried out only for few months.
2. Only one test fungus was taken.
3. Study was concentrated only on the use of two essential oils.
5. Different political hindrances during the period of experimentation.

2. LITERATURE REVIEW

Research works on the control of *Fusarium moniliforme* by using essential oil and extracts of aromatic plants and other related works are mentioned here.

2.1 Essential oils

Gulati and Suri (1982) considered essential oils as luxurious items because of their traditional use as flavoring agents in perfumes, cosmetics, beverage, foods and confectionery. Few people, who are actively associated with commerce or research of essential oils are aware of their inherent antibacterial, antifungal, insecticidal and antihelminthic efficiency which makes them highly valuable as irritants, skin stimulants, expectorants, antiseptics, disinfectants, antihelminthical, antibacterial and antifungal agents.

Shrestha *et al.* (1994) collected two hundred and nineteen species of aromatic and medicinal plants belonging to sixty plant families from different parts of the country and essential oil content of these plants were determined by hydro distillation method.

Isman (2000) studied the utility of essential oils of aromatic plants and analyzed their possible herbicidal activities. He mentioned that these essential oils could be the good herbicides since they do not persist in soil or contaminate ground water and cause little or no mammalian toxicity.

Fandohan *et al.* (2004), from their analysis reported that several compounds were present in the essential oil, of which monoterpenes were predominant and also found that oil from *Cymbopogon citratus* contained citral (neral and geranial) 47% and myrcene28% where as oil from *Eucalyptus citriodora* contained mainly citronellal 66% along with a small amount of the alcohol citronnellol 12%.

2.2 Antifungal activities of essential oils

Singh *et al.* (1980) found that essential oil from *Cymbopogon martini*, *C.oliveri* and *Trachyspermum ammi* exhibited strong antifungal activity against *Helminthosporium oryzae*.

Asthana *et al.* (1982) studied the volatile activity of 20 plant species against *Helminthosporium oryzae.* The extracts of *Ageratum conyzoides*, *Cymbopogon martini var. motia*, *Eupatorium capillifolium* and *Ocimum adscendens* exihibited absolute toxicity. The volatile antifungal constituents isolated as essential oils inhibited the mycelial growth completely at their minimum inhibitory concentrations of 200 µl / l for *Ocimum adscendens*, 250 µl / l for *Ageratum conyzoides* and 1000 µl / l for rest of others. The oils were non toxic to paddy and checked the appearance of disease symptoms during *in vivo* trials.

Tripathi *et al*. (1983) reported the essential oil of rhizome of *Alpina galangal* exhibited the highest toxicity to the mycelia growth of *Helmithosporium oryzae*. The volatile oil was fungi static at MIC of 0.4% in the medium. The oil controlled brown spot disease on detached paddy leaves and had no adverse effect on seed germination and growth of paddy seedlings.

Pattnaik *et al.* (1996) had taken essential oils of different plants such as Lemon grass, Eucalyptus, Orange, Geranium, Palmorosa etc and were tested for antibacterial activity against 22 bacteria and 12 fungi .they found out the minimum inhibitory concentration (MIC) of Eucalyptus, Lemon grass, Palmorosa and Peppermint oils ranged from 0.16 to >20µml^{-1} for eighteen bacteria and from 0.25 to10 µl/ ml for twelve fungi.

Baruah *et al.* (1996) studied effect of essential oils against *Fusarium verticilliodes*. They also observed that oil from *Eucalyptus citriodora* was less effective in its antifungal activity than that of the oil from *Cymbopogon* spp.

Kothavade *et al.* (1997) reported the appreciable antifungal activity of Cajupat oil, Eucalyptus oil and *Myristica fragrans* on different fungal diseases of plants.

Wilson *et al.* (1997) studied the rapid evaluation of plant extracts and essential oils for antifungal activity against *Botrytis cinerea.* They tested the fungi by extracts of 345 plants and essential oils from 49 plants and reported that the extracts from species of *Allium* and *Capsicum* and essential oils from *Cymbopogon martini and Thymus zygis* showed greatest antifungal activities.

Rai *et al.* (1999) studied *in vitro* susceptibility of opportunistic *Fusarium spp.* to essential oils. They reported that the essential oils extracted from the *Eucalyptus* species markedly inhibited fungal growth where as *Prosopic cineraria* did not show inhibitory properties.

Fiori *et al.* (2000) studied antifungal activity of leaf extracts and essential oils of some medicinal plants against *Didymella bryoniae.* They reported that the crude extracts of *Eucalyptus* and *Ageratum* species showed inhibitory effect on mycelia growth and germination of spore where as essential oil of *Cymbopogon citratus, Ageretum conizoids* and *Eucalyptus citriodora* provided 100% inhibition of the mycelia growth and germination of spores of *Didymella bryoniae*.

Bankole *et al.* (2002) studied the potentiality of powder and essential oil from dried ground leaves of *Cymbopogon citratus* (lemon grass) to control storage deterioration and aflatoxin contamination of melon seeds. Four mould species: *Aspergillus flavus*, *A. niger*, *A. tamarii* and *Penicillium citrinum* were inoculated in the form of conidia suspension into shelled melon seeds. The essential oil at 0.1 and 0.25ml\100g seeds and ground leaves at 10g\100g seeds significantly reduced deterioration and aflatoxin production in shelled melon seeds treated inoculated with toxigenic *A.flavus.* At higher dosage (0.5 and 1ml\100g seeds), the essential oil completely prevented aflatoxin production.

UF Researchers (2004) reported similarity between the essential oils and most soil fumigants. Essential oils products have a powerful smell because they are highly volatile and many of them vaporize quickly even at room temperature.

Lopez *et al.* (2004) studied the activities of different plant essential oils on *Fusarium verticilliodes,* fumonisin B1 production in corn grain. They reported that fumonisin production could be exhibited or stimulated by some constituents of essential oils coming from aromatic plants.

Nguefack *et al.* (2004) investigated the effect of essential oils extracted from *Cymbopogon citratus, Monodora myristica, Ocimum gratissimum,Thymus vulgaris and Zinziber officinale* against three food spoilage and mycotoxin producing fungi, *Fusarium moniliforme, Aspergillus flavus and Aspergillus fumigatus.* They reported that the essential oils from *Ocimum gratissimum, Thymus vulgaris ,Cymbopogon citratus* had effectively prevented conidial germination and the growth of all three fungi on corn meal agar at 800, 1000 and 1200ppm respectively.

Fandohan *et al.* (2004) evaluated *in vitro* and *in vivo* efficacy of essential oils from some local plants in Benin and neem seed against *Fusarium verticilliodes* infection and Fumonisin contamination.Oils from *Cymbopogon citratus, Ocimum basilicum and Ocimum gratissimum* were the most effective *in vitro* completely inhibiting the growth of fungi over 21 days of incubation. These oils reduced the incidence of pathogen in corn and totally inhibited fungal growth at concentration of 8.0, 6.4 and 4.8 µl ml^{-1}.

Parajuli R.R. (2005) studied inhibitory activity of essential oils from *Artemisia dubia, Artemisia gmelinni, Thymus linearis, Nardostachys grandiflora, Juniperus recurva and Zanthoxylem armatum* against *Alternaria brassicola. He* reported highest fungitoxicity of *Artimesia dubia Thymus linearis Juniperus recurva* at 10000, 5000 and 25000 ppm concentrations respectively.

Manandhar A. (2005) investigated antifungal activities of essential oils from six plants against *Bipolaris sorokiniana.* she reported appreciable inhibitory effects towards the growth of colony of fungi. Among six plants, essential oil of *Thymus linearis* was found to be the most effective in its fungitoxic properties.

Khaddor *et al.* (2006) had investigated antifungal activities of three essential oils on growth and toxigenesis of *Penicillium aurantiogriseum* and *Penicillium viridicatum*. A complete inhibition of toxin production was observed with 0.44% of each essential oil for *P.aurantiogriseum* and 0.22% for *P. viridicatum*.

Helal *et al.* (2006) studied the effects of essential oil of *Cymbopogon citratus* on the growth, lipid content and morphogenesis of *Aspergillus niger*. They determined the ultra structural modification of *A. niger* hyphae after treatment with *C. citratus* essential oil. They reported that the oil causes decreased hyphal wall and also caused plasma membrane disruption, mitochondrial structure disorganization and decreased in lipid content.

Helal *et al*. (2007) studied the effects of essential oil of *Cymbopogon citratus* on the growth, morphogenesis and alfatoxin production of *Aspergillus flavus*. They reported decreased in hyphal wall of fungi. They also found disruption of plasma membrane and disorganization of mitochondrial structure of *Aspergillus flavus*.

2.3 Antifungal activity of plant extract

Ark and Thompson (1959) showed that garlic extracts contain potent fungicides. They were able to effectively protect peaches against brown rot (*Monilinia fructicola*) with deodorized garlic extract preparations.

Rai *et al*. (2002) studied the effect of different plant extracts on *Fusarium monoliforme* reported the extracts of *Thuja, Vinea,* lower dosage of *Cinnamomum* oils and higher dosage of clove oil exhibited the inhibition of *Fusarium moniliforme.*

Lee *et al*. (2007) studied effects of some Chinese medicinal plant extracts on five different fungi that are *Aspergillus niger, Fusarium moniliforme, Botrytis cinerea,Glomerella cingulata* and *Phyllostica caricae.* They reported that the combination of herb extracts showed higher inhibitory effect towards tested fungi than that of individual extract.

3. MATERIALS AND METHODS

3.1 Materials

Different equipments, glass wares and chemicals were used in performing the research work. All the materials used are listed in the appendix-I.

3.2 Methods

3.2.1 Extraction of essential oils

Leaves of *Cymbopogon citratus* and *Eucalyptus citriodora* collected from Kirtipur were shade dried for three days. Fifty grams of these shade dried leaves were surface sterilized with 0.1% sodium hypochlorite solution and washed thoroughly with distilled water. The essential oils were then extracted from those leaves by hydrodistillation method using Clevenger's oil extracting apparatus. The oil collected was then dehydrated over anhydrous sodium sulphate and stored at 10° C.

3.2.2 Media preparation

Potato Agar Dextrose (PDA) media was prepared for culture of test fungal pathogen. For preparing 500 ml PDA media, 100 gm of finely peeled and then cut potato pieces were boiled in water till the pieces were soft to touch. The juice of boiled potato was filtered with muslin cloth into the conical flask. Agar and Dextrose each 10 gm were added and stirred. Final volume was made 500 ml adding distilled water. The solution was autoclaved for 25-30 minutes at 15 lb. pressure and 121° C temperature to get sterilized PDA media.

3.2.3 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 Petridishes 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.

3.2.4 Experiments

The effect of essential oils from *Cymbopogon citratus* and *Eucalyptus citriodora* on the test fungus (*Fusarium moniliforme*) from rice was analyzed in two sets viz. 1st. and 2nd. In 1st set, the mycelial growth in *Fusarium* discs (from its 7th. day old pure culture) on PDA media poisoned with six different concentrations (with 1 control group) of both essential oils ranging from 1.2 - 12.4 $\mu l\, ml^{-1}$ was assessed making three replicas for each concentration. Similarly, in 2nd set, the *Fusarium* inoculated rice seeds were treated in 11 different concentrations of both essential oils ranging from 0.04 - 1.4 ml/ gram (with 2 control groups) and plotted in plain PDA media making 2 replicas for each concentration.

1st set

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 $\mu l\, ml^{-1}$ of 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 100 µl of ethanol plus 1ml of water and mixing with 15ml of melted sterile PDA.

Each concentration of oil was poured into separate Petridishes with three replicas and mixed with 15 ml of PDA media. Each Petridish 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 Petridishes were incubated for 20 days at 25° C.

Three Petridishes 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.

2^{nd} set

In the experiment two hundred and sixty autoclaved rice grains were artificially inoculated with 2 ml of conidial suspension of *Fusarium moniliforme.* The conidial suspension was prepared by adding 5 ml of sterile distilled water to a seventh day old culture of test pathogen. The culture was superficially scraped to free the conidia from conidiophores and conidial suspension was filtered through muslin cloth. The inoculated rice grains were then treated with oils from *Cymbopogon citratus* and *Eucalyptus citriodora* at their different concentrations obtained by diluting 20, 40, 60, 80, 100, 200, 300, 400, 500, 600 and 700 µl of each oil with 100 µl of 95% ethanol and 1 ml sterile distilled water separately. Twenty rice seeds (0.5g) were allowed to soak in each concentration of each oil for 20 minutes and then dried for 10 minutes and plated into Petridishes containing PDA media. The concentration range of each oil per rice seed were therefore 0.04, 0.08, 0.12, 0.16, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.4 ml / g. Ten grains were plotted in each Petridish making two replicas for each concentration. The Petridishes were incubated at 25° C. Antifungal activity of oil was assessed after 7 days by counting the number of infected and non infected rice grains in each Petridish and calculating the healthy grain percentage (*i.e.* of non infected grain per oil concentration). There were two controls, one –Rice grain inoculated with *Fusarium moniliforme* and treated with a solution of 100 µl of 95% ethanol plus 1ml of sterile distilled water and another-Rice grain inoculated with *Fusarium moniliforme* with no further treatment.

3.2.5 Calculations

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

3.2.6 Measurement of conidia

Ocular micrometer was placed inside the eye piece of a compound microscope and calibrated with the help of stage micrometer. The number of division of ocular micrometer coinciding with the number of division in stage micrometer was noted and calculated the calibration factor applying the formula.

$$\text{One ocular division} = \frac{\text{No. of division on stage micrometer}}{\text{No. of division on ocular micrometer}} \times 10\mu m$$

Then, the stage micrometer was replaced by a fine slide of *Fusarium moniliforme*. Size of conidia was measured by determining the number of ocular micrometer (appendix-II) Measurement was carried out to find the range of size of conidia.

3.2.7 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. Spss 11.5 windows version was used for analytical statistics.

4. RESULTS

4.1 Effects of essential oils on mycelium growth of *F. moniliforme*

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 $15^{th.}$ and $20^{th.}$ 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. In other words the size of the fungal colony decreased along with increase in concentrations of essential oils used. 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 10^{th}. to 20^{th}. days.

Percent inhibition in colony size of the test fungus due to *Eucalyptus* oil at 10^{th}, 15^{th}, and 20^{th} day of incubation is shown in Appendix II, Table no. 1, 2 and 3 respectively. Similarly, Percent inhibition in colony size of the test fungus due to *Cymbopogon* oil at 10^{th}, 15^{th}, and 20^{th} day of incubation is shown in Appendix II, Table no. 4, 5 and 6 respectively.

These findings were plotted in representible bar diagrams which are shown sequentially one after another from figure-1 to 6. 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 7 and 8 respectively.

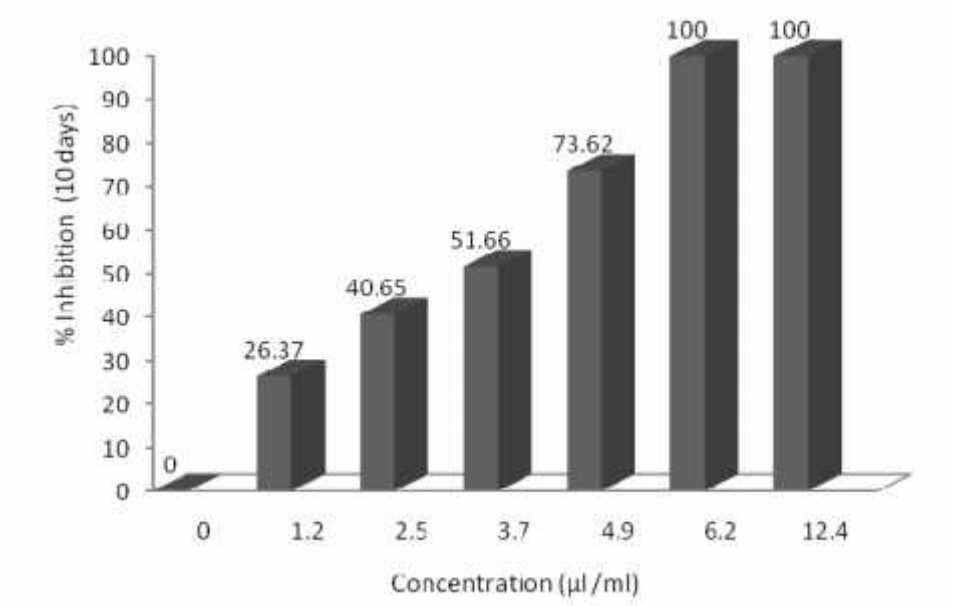


Figure 1- Percent inhibition of mycelial growth of test fungus at different concentrations of *Eucalyptus* oil after 10 days of incubation.

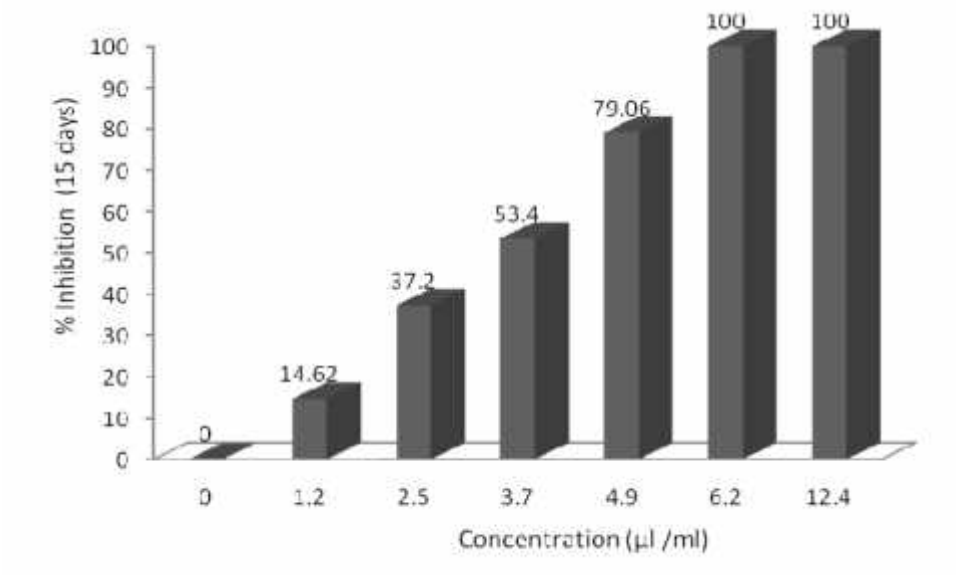


Figure 2- Percent inhibition of mycelial growth of test fungus at different concentrations of *Eucalyptus* oil after 15 days of incubation.

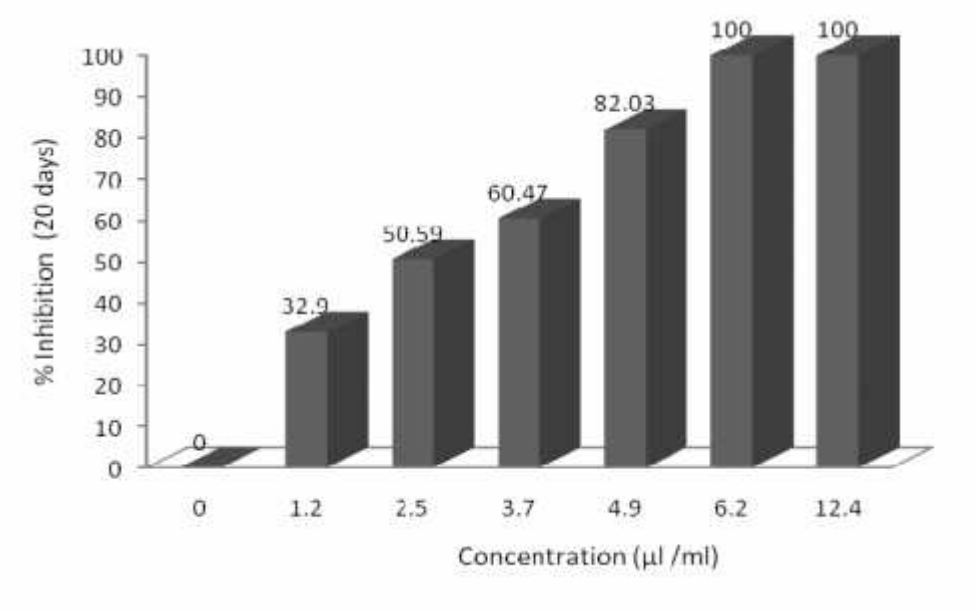


Figure 3- Percent inhibition of mycelial growth of test fungus at different concentrations of *Eucalyptus* oil after 20 days of incubation.

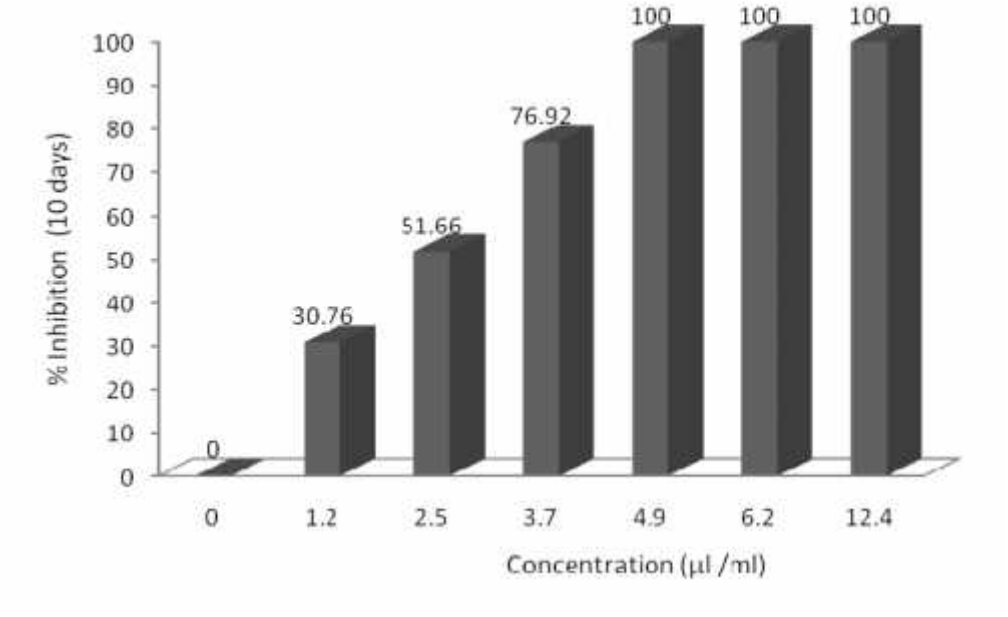


Figure 4- Percent inhibition of mycelial growth of test fungus at different concentrations of *Cymbopogon* oil after 10 days of incubation

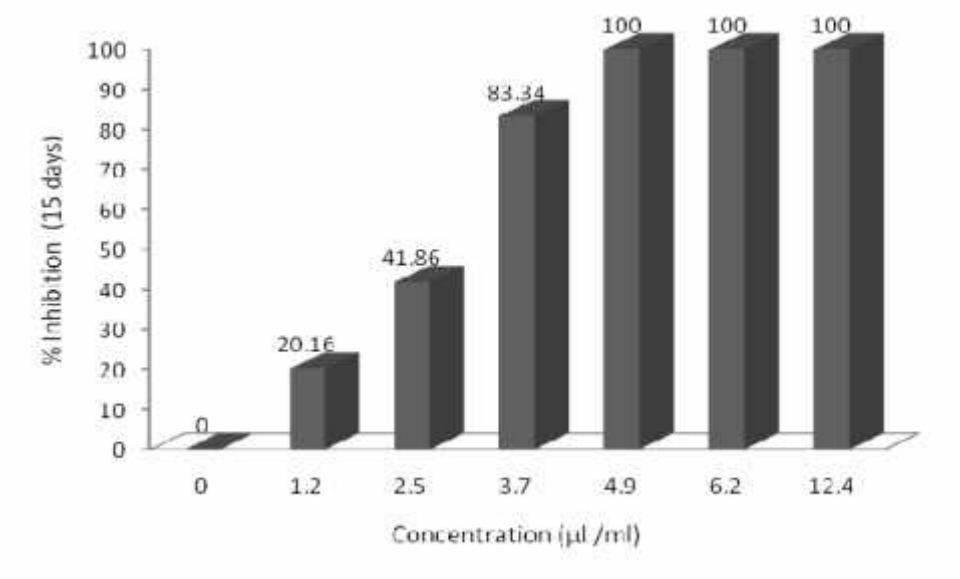


Figure 5- Percent inhibition of mycelial growth of test fungus at different concentrations of *Cymbopogon* oil after 15 days of incubation.

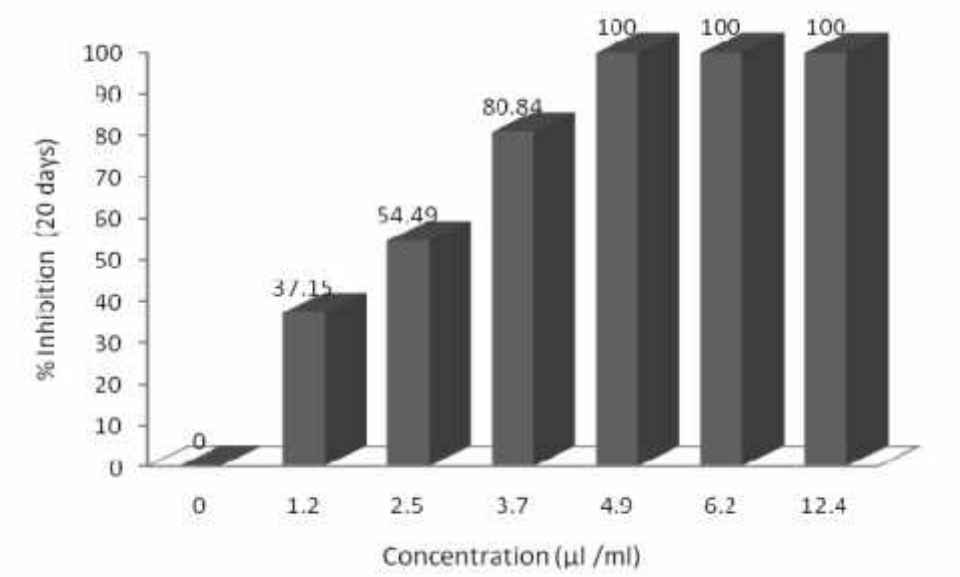


Figure 6- Percent inhibition of mycelial growth of test fungus at different concentrations of *Cymbopogon* oil after 20 days of incubation.

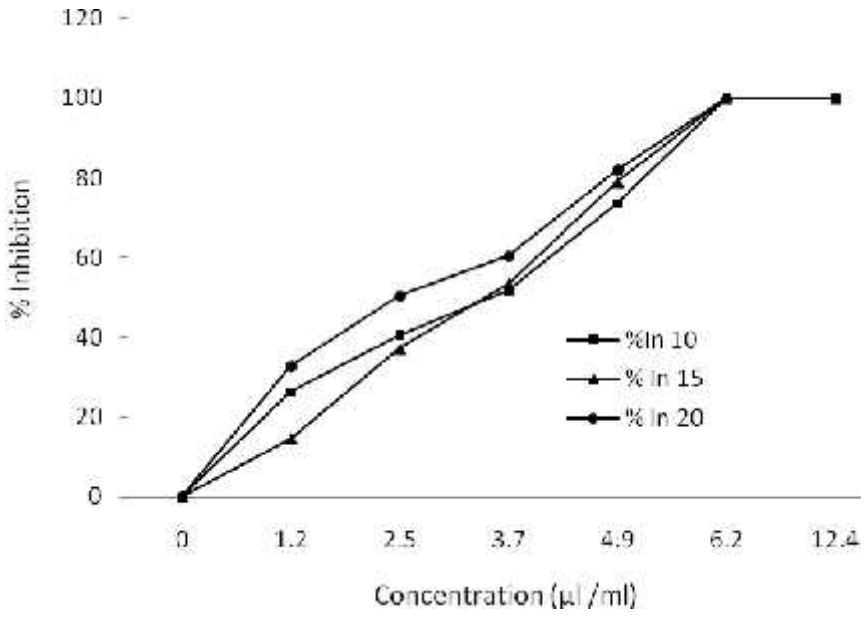


Figure 7- Comparative graph of % inhibition in varying concentrations of *Eucalyptus* oil at10, 15 and 20 days of incubation.

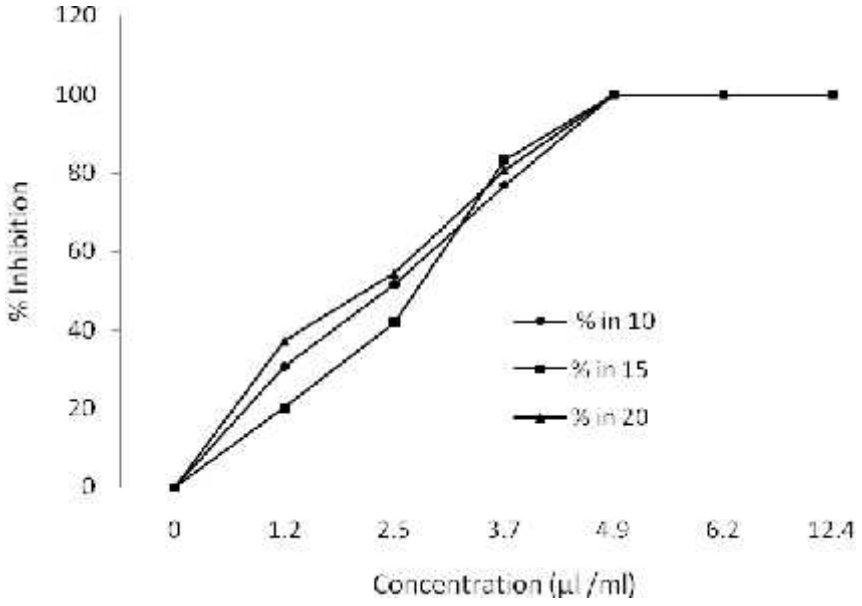


Figure 8- Comparative graph of % inhibition in varying concentrations of *Cymbopogon* oil at10, 15 and 20 days of incubation.

The comparative graph shown on figure 7 and 8 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: ANOVA for colony size at different oil concentrations

Source of Variation	Sum of square	Degree of freedom	Mean square	Variance Ratio
Total	4421.2	13	-	-
Treatment	4346	6	724.33	98.41^{**}
Plants	31.03	1	31.03	4.21^{ns}
Residual mean square	44.17	6	7.36	

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

The comparative effect of both essential oil on mean colony size at 20^{th}. day of incubation is shown in the figure – 9 which indicates the gradual decrease in the mean colony size of test fungus with the increase in concentrations of both essential oils. The general effect of essential oils on the mycelial growth of the test fungus is shown on figure -10 which indicates sharp declination in the average mean colony size along with the increase in concentration of each essential oil from 0 to 6.2 µl ml^{-1} and complete inhibition is seen at and above 6.2 µl ml^{-1}concentration.

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.

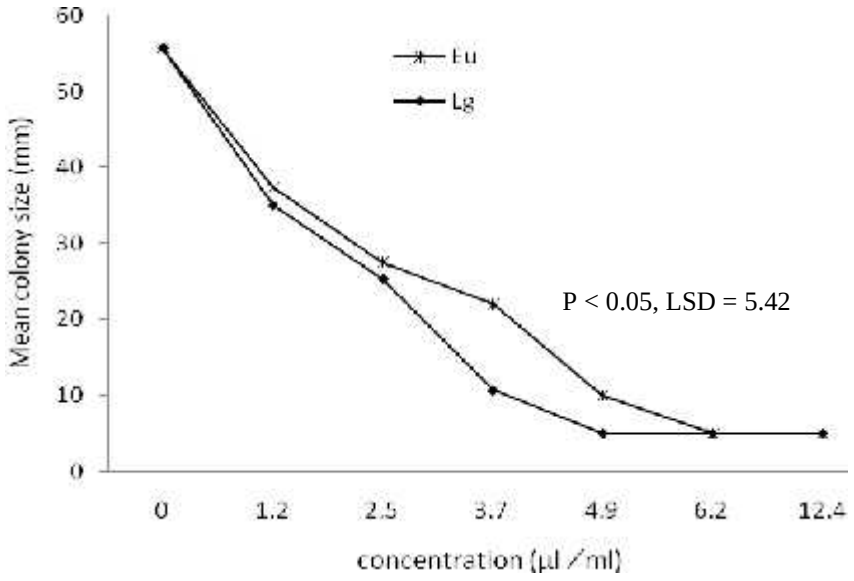


Figure 9- Effect of different concentration of two different oils on the colony size of test fungus.

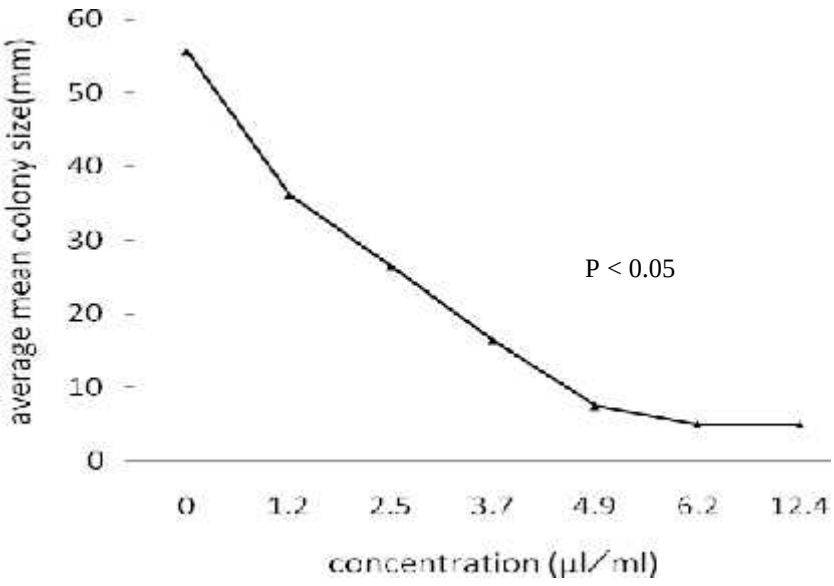


Figure 10 - General effect of essential oils on the colony size of the test fungus.

The correlation analysis of mean colony size of test fungus against various concentrations of tested oil revealed the correlation factors - 0.841, - 0.845, - 0.823 for *Eucalyptus* oil at 10, 15 and 20 days of incubation respectively. Similarly, the correlation factors for *Cymbopogon* oil were – 0.745, - 0.759 and - 0.755 at 10, 15 and 20 days of incubation respectively. The table – 8 and figure – 11 shows the comparative correlation factors of both essential oils against colony size of *Fusarium moniliforme.*

Table - 2: Correlation analysis of mean colony size of *Fusarium moniliforme* with various concentrations of *Eucalyptus* and *Cymbopogon* oil.

S. N.	**Incubation time (Days)**	**Correlation Factor of MCS against *Eucalyptus* Concentrations**	**Correlation Factor of MCS against *Cymbopogon* Concentrations**
1	10	- 0.841**	- 0.745
2	15	- 0.845**	- 0.759**
3	20	- 0.823**	- 0.755**

** = correlation is significant at 0.05 levels

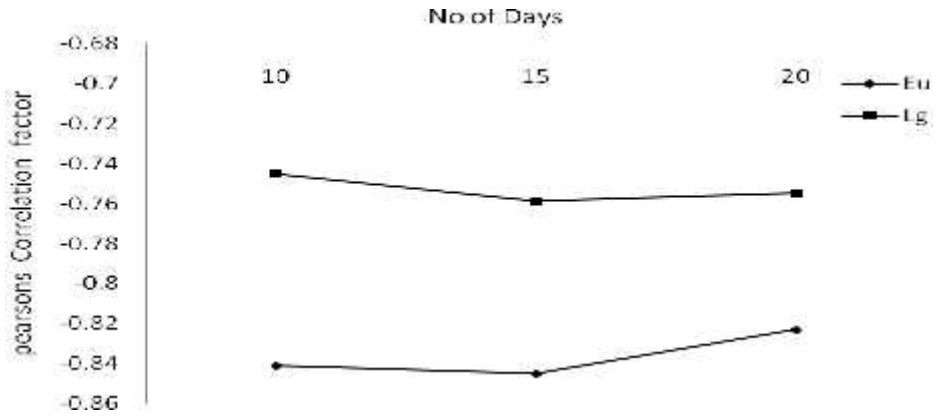


Figure 11- Comparative graph of correlation factors of mean colony size of test fungus against various concentrations of both oils.

4.2 Effects of essential oils on Rice seed contamination

Rice seed infection by test fungus after the treatment by essential oils from *Eucalyptus citriodora* and *Cymbopogon citratus* at their different concentrations ranging from 0, 0.04, 0.08, 0.12, 0.16, 0.2, 0.4, 0.8, 1, 1.2, and 1.4 ml g^{-1} was analyzed. Different concentrations of both oils could not completely inhibit the fungal contamination on the rice seeds. However, the maximum inhibition was achieved at concentration 1.4 ml g^{-1} of *Cymbopogon* as well as *Eucalyptus* oil reaching to the value 90% and 75% respectively. The table -9 and 10 and corresponding graph (Fig. 12) below shows the effect of both essential oils on % inhibition of fungal contamination on the oil treated rice seeds

Table - 3: Infection of test fungus to oil (*Eucalyptus*) treated rice seeds on 7$^{th.}$ day of incubation.

S. N.	**Concentration of oil (ml g^{-1})**	**Total no. of seeds**	**Infected seeds**	**Non infected seeds**	**Inhibition (In %)**
1	0	20	20	0	0
2	0.04 - 0.8	20	20	0	0
3	1	20	14	6	30
4	1.2	20	10	10	50
5	1.4	20	5	15	75

Table - 4: Infection of test fungus to oil (*Cymbopogon*) treated rice seeds on 7$^{th.}$ day of incubation.

S. N.	**Concentration of oil (mlg^{-1})**	**Total no. of seeds**	**Infected seeds**	**Non infected seeds**	**Inhibition (In %)**
1	0	20	20	0	0
2	0.04 - 0.4	20	20	0	0
3	0.6	20	13	7	35
4	0.8	20	12	8	40
5	1	20	7	13	65
6	1.2	20	5	15	75
7	1.4	20	2	18	90

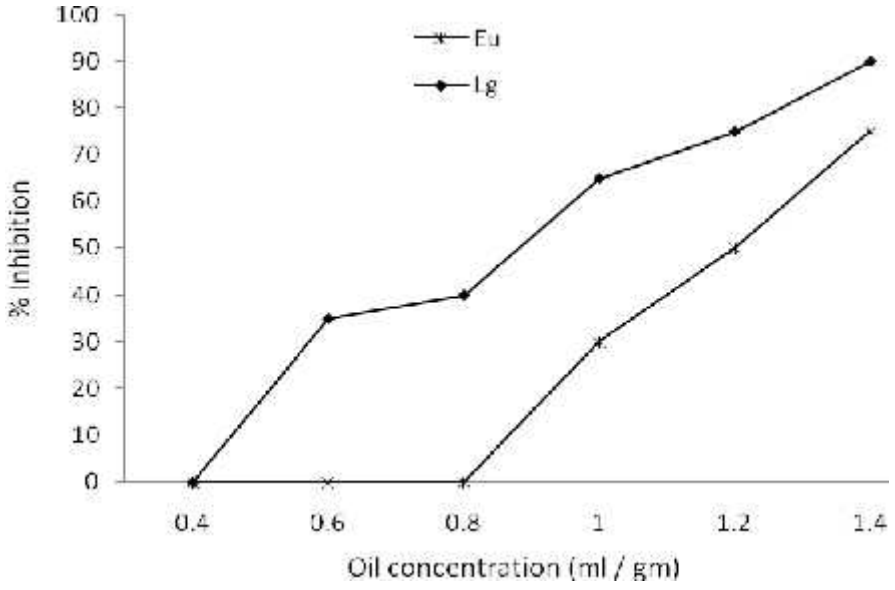


Figure 12- Effect of essential oils on *Fusarium moniliforme* contamination of rice seeds.

5. DISCUSSION

Since the discovery of antifungal and microbial properties, preparation of essential oils have been applied in pharmacology, medical microbiology, phytopathology and food preservation (Magiatis *et al.* 2002). They are formed into the plant itself on various locations such as flowers, leaves, roots, wood, barks, seeds and resin etc. The use of essential oils to control post harvest fungi and pests is gaining attention because of the increasing public concern over the level of pesticide residues in food (Bishop *et al*. 1997). Essential oils are also less likely to be associated with the development of resistance than is the case with fungicides and are less hazardous to the environment and human health than synthetic pesticides (Daferera *et al*. 2003). Besides, essential oils can be used as good herbicides since they are highly volatile and do not persist in soil for long time and therefore have little or no mammalian toxicity (Isman, 2000).

In present study the essential oils used showed variable antifungal activities at different concentrations against *Fusarium monoliforme*. Both the essential oils used were found significantly ($p < 0.05$) affecting the growth of *Fusarium moniliforme* at and after 10^{th}.day of incubation thereby arresting the mycelial growth of the test fungus. The reported effective concentrations of *Eucalyptus citriodora* and *Cymbopogon citratus i.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 *Fusarium moniliforme* in this research is also supported by Baruah *et*

al., 1996 with almost same findings. They also found *Cymbopogon* spp. more effective than *Eucalyptus citriodora* in arresting mycelial growth of *Fusarium.*

The reason for antifungal activities of both essential oils may be attributed to their chemical compositions. Different biochemical studies of *Cymbopogon* extract revealed the presence of citral (neral and geranial) and myrcene as chief components. Similarly, the oil of *Eucalyptus* contained mainly citronnellal and citronnellol as chief components (Fandohan *et al.*, 2004). The presence of these monoterpenes and diterpenes in the plant essential oils might have some sort of antagonistic behavior during the mycelial growth of the test fungus since they are reported to have different cytotoxic potential (Dayan *et al.*, 1999). Helal *et al.* reported that the oil of *Cymbopogon citratus* causes decreased hyphal wall, plasma membrane disruption, mitochondrial structure disorganization and decreased in lipid content on *Aspergillus niger* (in 2006) and on *A. flavus* (in 2007). These essential oils are also reported for affecting adversely on fumonisin production by *Fusarium moniliforme* (Fandohan *et al.*, 2004). High degree of inhibitory effect of used essential oils against mycelial growth of test fungus was also supported by the negative correlation factors lying between -0.7 to -0.9. The inhibition in the infection by test fungus on rice seeds might be due to the similar reasons as described earlier.

The results from the study may not be true forever because the concentration of various ingredients in essential oils of same plant species varies due to several factors such as growth stage, ecological and environmental factors etc. Also the chemical characterization of the used essential oils and their practical implications are yet to be tested. Similarly, the possible role of these terpenes in regulating the fungal growth precursors can not be overruled. Hence, further researches in the line of chemical characterization of used essential oils and their field trial experiments against *Fusarium moniliforme* are speculated to justify the present findings.

6. CONCLUSION AND RECOMMENDATIONS

6.1 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*.

3) Low concentrations of these essential oils are highly effective to inhibit the mycelial growth completely from pure culture disc of *Fusarium moniliforme*. However, *Fusarium moniliforme* inoculated rice seeds require high concentrations of these essential oils to arrest the mycelial growth of the same pathogen in laboratory conditions.

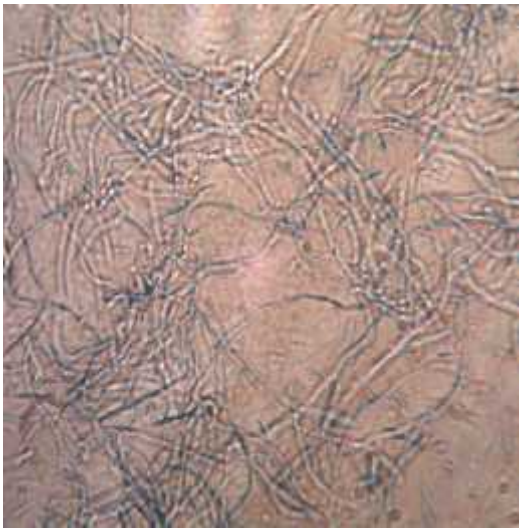
6.2 Recommendations

After the overall study following postulates are recommended:

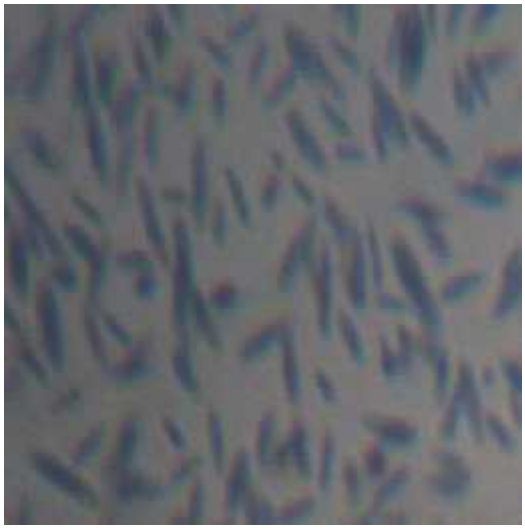
1) The field trial experiments of these essential oils as bio fungicide are emphasized to carry out.

2) The researches in the line of chemical characterization of used essential oils and their possible role as biological fungicides along with the mechanism of inhibition is speculated to justify the present findings.

PHOTOPLATE – I



Mycelium and microconidia



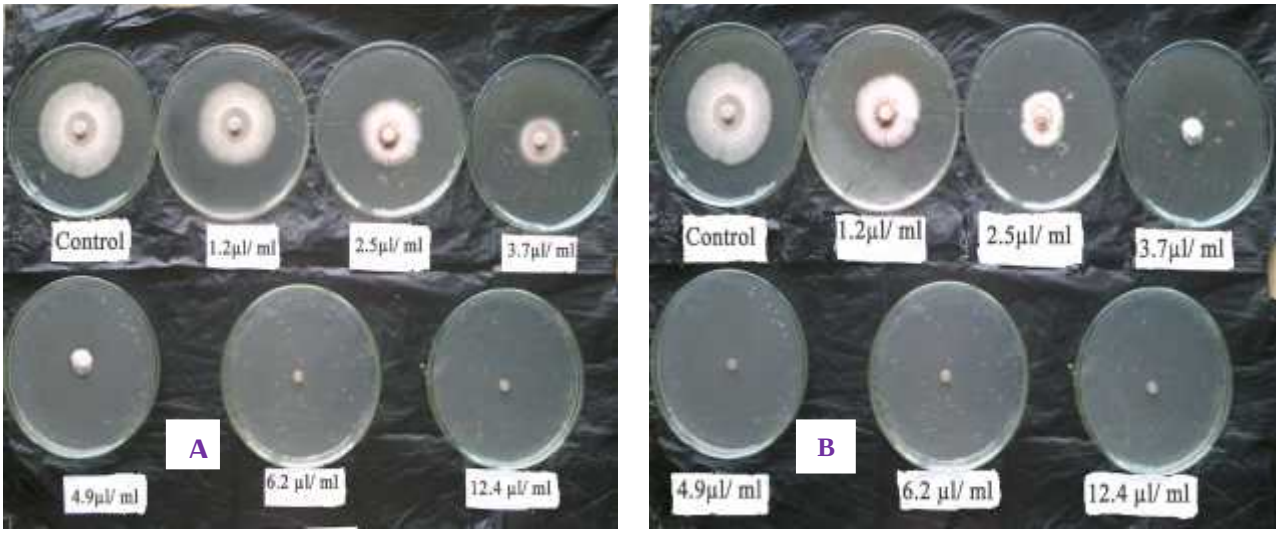
Macroconidia under high power

Herbarium specimen of *Cymbopogon Citratus*

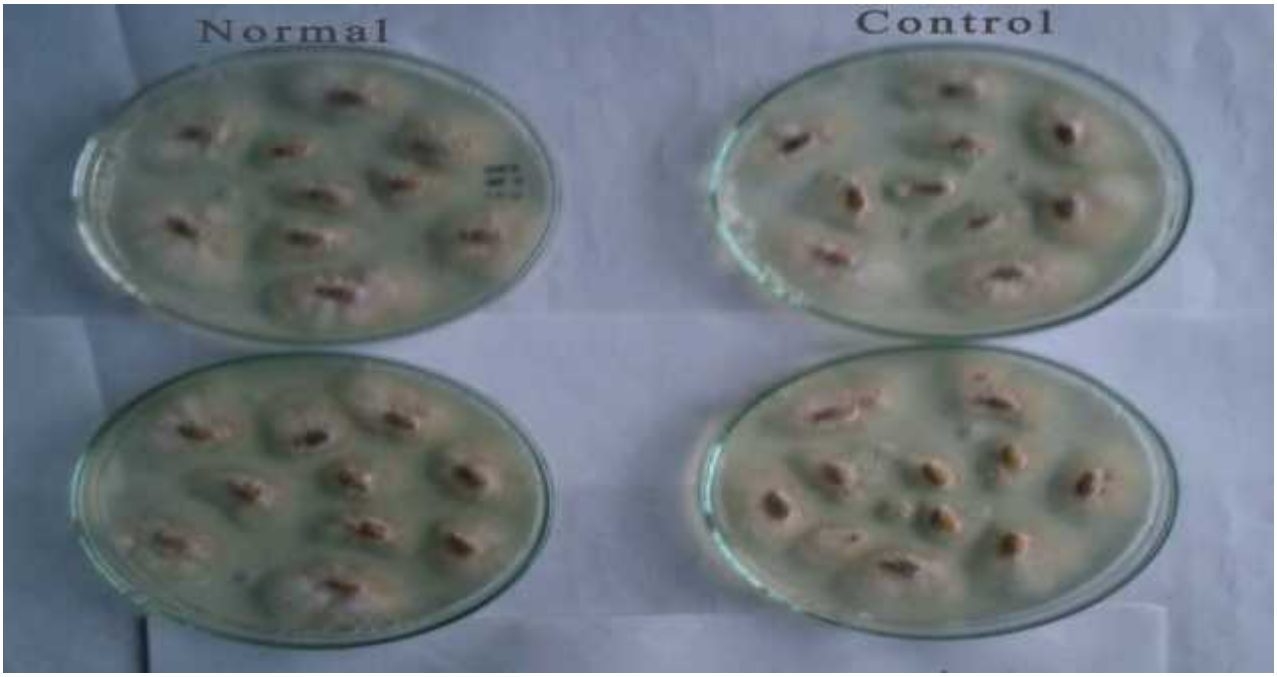
Herbarium specimen of *Eucalyptus citriodora*

PHOTOPLATE – II

Time: at 20th. day of incubation



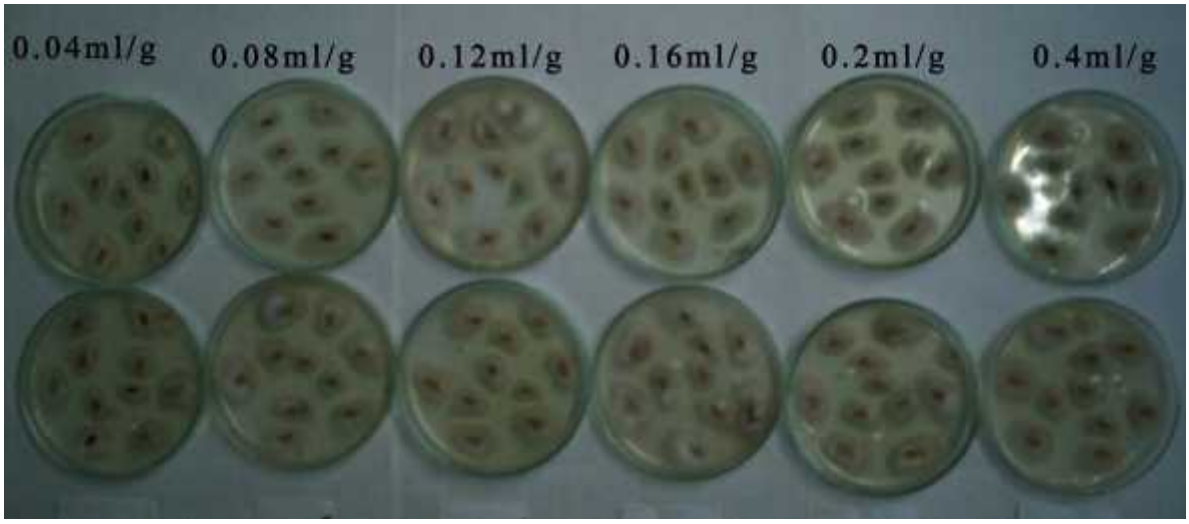
Effect of *Eucalyptus citriodora* (A) and *Cymbopogon citratus* (B) oils at varying concentrations (0 – 12.4µl / ml) on mycelium growth of *Fusarium moniliforme*.



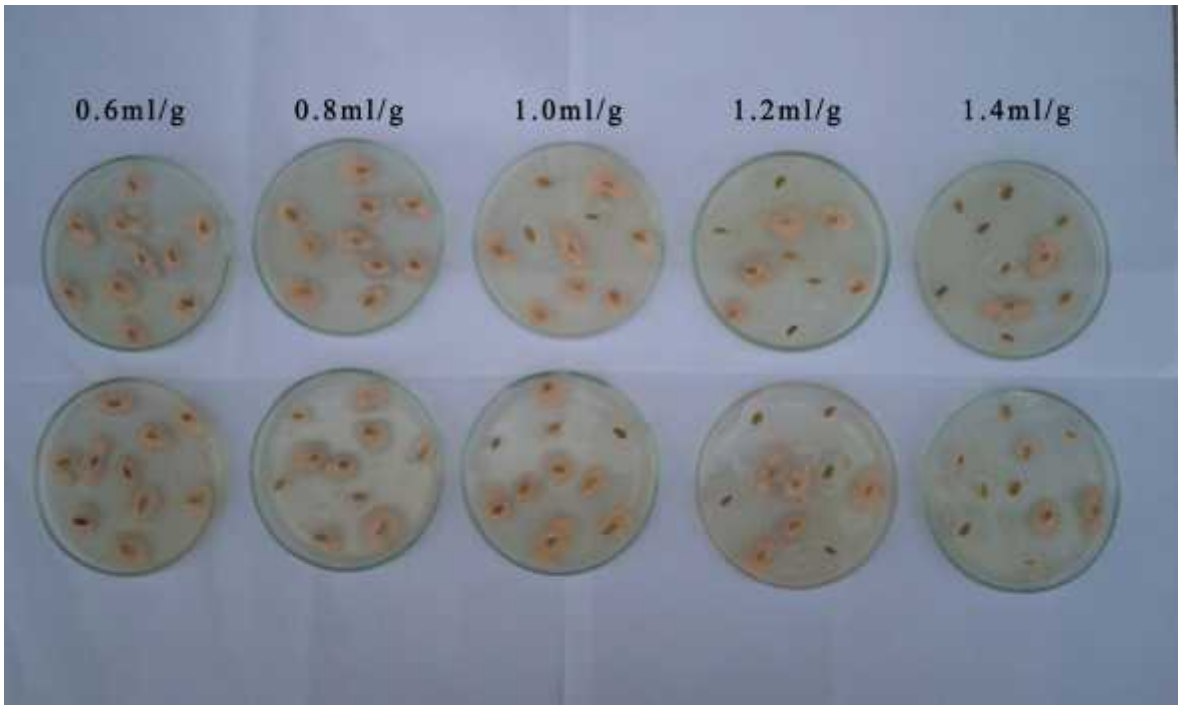
Contamination of *Fusarium moniliforme* in rice seeds at Normal and control groups at 7$^{th.}$day of incubation.

PHOTOPLATE – III

Time: at 7$^{th.}$day of incubation



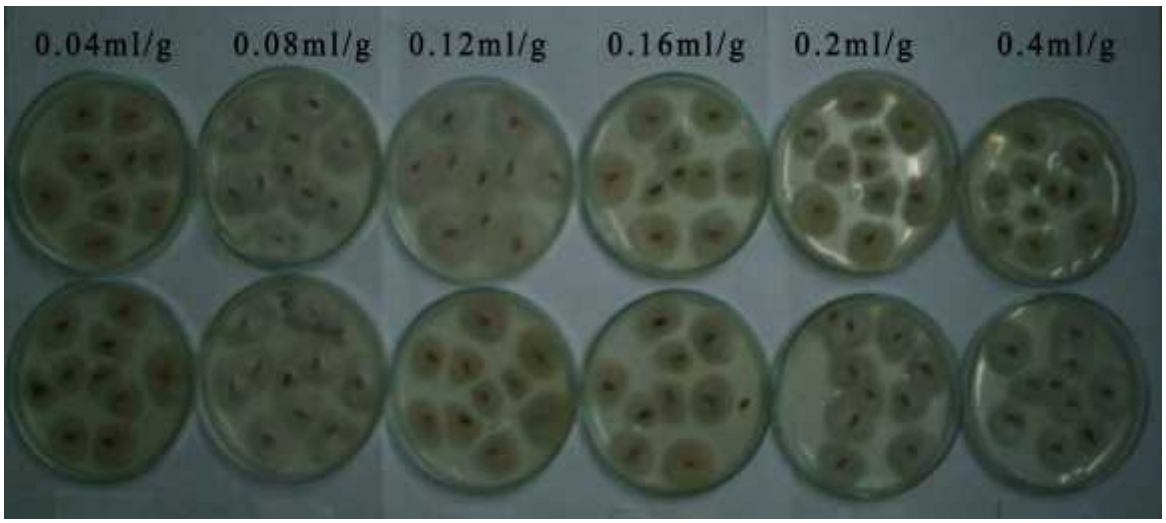
Effect of Eucalyptus oil at varying concentrations (0.04 - 0.4 ml /g) on rice seed contamination by *Fusarium moniliforme.*



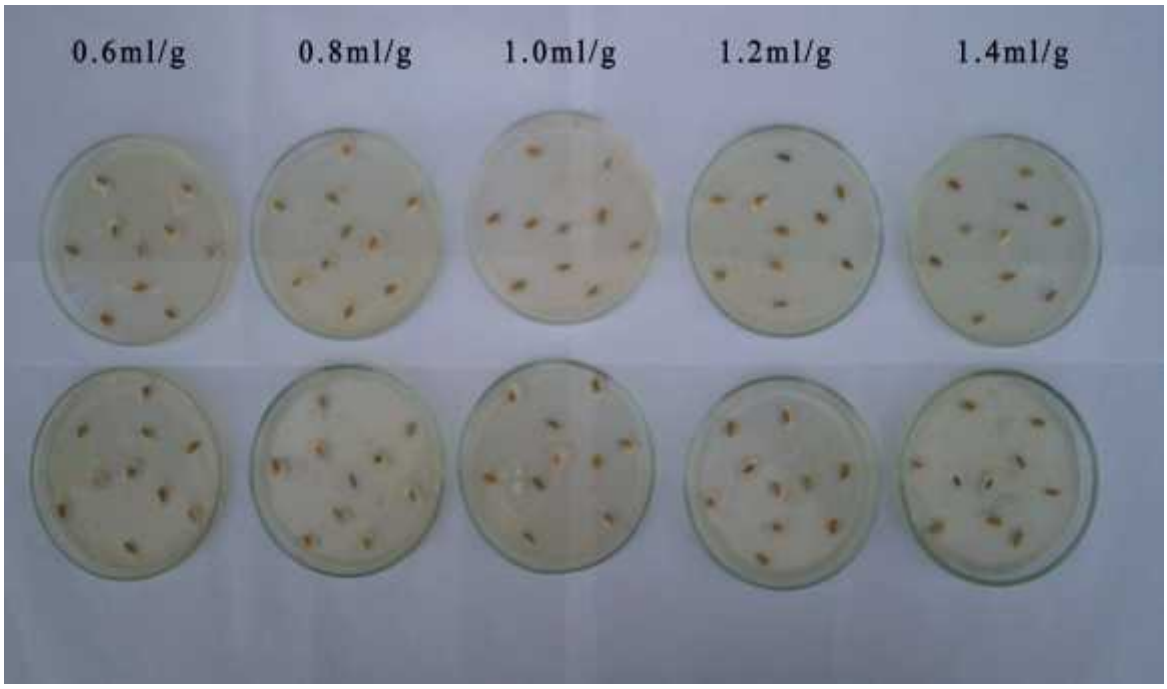
Effect of Eucalyptus oil at varying concentrations (0.6 - 1.4 ml /g) on seed contamination by *Fusarium moniliforme.*

PHOTOPLATE – IV

Time: at 7$^{th.}$day of incubation



Effect of Lemon grass oil at varying concentrations (0.04 - 0.4ml /g) on rice seed contamination by *Fusarium moniliforme.*



Effect of Lemon grass oil at varying concentrations (0.6 - 1.4ml /g) on rice seed contamination by *Fusarium moniliforme.*

Appendix I

A. Materials used for the study

1. Essential oils

Eucalyptus citriodora and *Cymbopogon citratus*

2. Media

Potato dextrose agar

3. Chemical and reagent

95% ethanol, Sprit, Sterilized water, Sodium hypochloride, Anhydrous sodium sulphate

B. Apparatus and equipment

Test tubes, Conical flasks, Beakers, Petridishes, Pipettes, Measuring cylinder, Glass slides, Cover slips, Inoculating loop, Forceps, Borers, Measuring scales, Autoclave, Hot air oven Incubator, Heater, Refrigerator, Laminar air flow, Microscope, Ocular micrometer, Stage micrometer etc.

Appendix II

Table -1: Colony size on10$^{th.}$ day of incubation in varying concentration of *Eucalyptus* oil.

S. N.	Inoculum size (mm)	Con.^{n} of oil (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

S. N.	Inoculum size (mm)	Con.^{n} of oil (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 - 2: Colony size on15$^{th.}$ day of incubation in varying concentration of *Eucalyptus* oil.

Table – 3: Colony size on 20$^{th.}$ day of incubation in varying concentration of *Eucalyptus* oil.

S. N.	Inoculum size (mm)	Con.^{n} of oil (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

S. N.	Inoculum size (mm)	Con.^{n}ofoil (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 - 4: Colony size on10$^{th.}$ day of incubation in varying concentration of *Cymbopogon* oil.

Table - 5: Colony size on 15$^{th.}$ day of incubation in varying concentration of *Cymbopogon* oil.

S. N.	**Inoculum size (mm)**	**Con.^{n}of oil (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

S. N.	**Inoculum size (mm)**	**Con.^{n} of oil (µlml^{-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

Table - 6: Colony size on 20$^{th.}$ day of incubation in varying concentration of *Cymbopogon* oil.

Appendix III

Micrometry

100 division of stage micrometer coincided with 54 division of ocular micrometer.

$$1 \text{ ocular division} = \frac{\text{No. of division in stage micrometer}}{\text{No. of division in ocular micrometer}} \times 10\ \mu m$$

$$= 100/54 \times 10\ \mu m$$

$$= 18.5$$

Table 1: Measurement of length and breath of macro conidia

S.N.	Calibration factor	No.of ocular div.(L)	No.of ocular div.(B)	Length of conidia	Breadth of conidia
1	18.5	2	.5	37	9.2
2	18.5	2.1	.5	38.8	9.2
3	18.5	2.2	.5	40.7	9.2

4	18.5	1.9	.5	35.1	9.2
5	18.5	1.7	.4	31.4	7.4
6	18.5	2	.5	37	9.2
7	18.5	2	.5	37	9.2

Size of conidia = 31.4 - 40.7 x7.4 – 9.2

Table 2: Measurement of diameter of micro conidia

S.N.	Calibration factor	No. of ocular div.	Diameter of conidia
1	18.5	.6	11.1
2	18.5	.6	11.1
3	18.5	.5	9.2
4	18.5	.5	9.2
5	18.5	.5	9.2
6	18.5	.6	11.1
7	18.5	.6	11.1

Size of conidia = 9.2 – 11.1

Appendix IV

Calculation for ANOVA

The statistical analysis test for ANOVA analysis was carried out using Microsoft excel. The results are tabulated below

Table – 1 percent inhibition of mycelial growth of test fungus by *Eucalyptus* and *Cymbopogon* oils.

Plants	**Control**	**1.2mlL^{-1}**	**2.5mlL^{-1}**	**3.7mlL^{-1}**	**4.9mlL^{-1}**	**6.2mlL^{-1}**	**12.4mlL^{-1}**	**Total**
Eu.	55.66	37.33	27.5	22	10	5	5	**162.49**
Lg.	55.66	35	25.33	10.66	5	5	5	**141.65**
Total	111.32	72.33	52.83	32.66	15	10	10	**304.14**
Average	**55.66**	**36.16**	**26.41**	**16.33**	**7.5**	**5**	**5**	**-**

No. of data=14, $\sum X=304.14$

Here, N=14, $\sum X=304.14$

a) Correction factor (C_f) = $(\sum X)^2 / N = (304.14)^2 / 14 = 6607.22$

b) Total sum of the squares about the mean (TSS) = $(n_1)^2 + (n_2)^2 + \ldots\ldots\ldots\ldots + (n_{14})^2 - C_f$

Where n = a single data

$= (55.66)^2 + (37.33)^2 + \ldots\ldots\ldots\ldots (10)^2 - 6607.22$

$= 11028.419 - 6607.22$

$= 4421.2$

c) Sum of square for treatment (SST) = $1/2\ [(111.32)^2 + (72.33)^2 + (52.83)^2 + (32.66)^2 + (15) + (10)^2 + (10)^2] - 6607.22$

$= 1/2\ (21906.45) - 6607.22$

SST = 4346

d) Sum of square of plants about mean (SSP) = $1/7\ [(1162.49)^2 + (141.65)^2] - 6607.22$

$= 1/7\ (46467.77) - 6607.22$

$= 31.03$

Hence,

Residual error = TSS – SST – SSP = 4421.2 – 4346 – 31.03 = 44.17

The treatments were found significant with variance ratio 98.41.

We analyse only the significant case.

Hence,

$$LSD = P_{0.05;\, df = 6} \times \sqrt{\frac{2 \times \text{Residual mean square}}{\text{No. of replications}}}$$

$$= 2.447 \times \sqrt{\frac{2 \times 7.36}{3}} \qquad [\text{Since, } P_{0.05;\, df = 6} = 2.447]$$

= 5.42 (approx.)

Hence,

Least significance difference (LSD) = 5.42

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