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Full Length Article

Lemongrass Essential Oil as an Alternate Approach to Manage Seed Associated Fungi of Wheat and Rice

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Abstract

Rice and wheat germplasm were vetted for the presence of seed both saprophytic as well as pathogenic fungi associated with wheat and rice seeds. Seven fungal species *viz.*, *Alternaria alternata*, *Aspergillus flavus*, *A. niger*, *Curvularia lunata*, *Drechslera indica*, *D. tripogonis* and *Fusarium moniliforme* were associated with rice while five fungi *viz.*, *A. alternata*, *A. flavus*, *A. fumigatus*, *A. niger* and *D. indica* were found in wheat. Among all the isolated fungi, *A. flavus* was found as predominant fungus (41%) associated with seeds of rice and (33%) with wheat seeds. The antifungal potential at different concentrations of lemongrass essential oil was tested by using disk diffusion method against isolated fungal species. Minimum inhibitory concentration (MIC) of lemongrass essential oil against each fungus was determined through agar plug method. Highest MIC (55 ppm) was recorded for *A. flavus* and lowest for *D. indica* (20 ppm). Significant differences were recorded for different concentrations of lemongrass essential oil against isolated fungi ($p \leq 0.05$). However, *A. niger* was found to be the most susceptible fungal strain to lemongrass essential oil. Results of the contemporary experiments indicate that lemongrass essential oil could be employed as alternate to synthetic fungicides during grain storage.

Keywords: Lemongrass essential oil; Seed associated fungi; Rice; Wheat; *Aspergillus niger*

Introduction

Rice (*Oryza sativa*) and wheat (*Triticum aestivum*) are the main food of Pakistani population. These plants are subjected to environmental stresses and numerous injuries at all stages of growth that interfere with their normal functioning and development. Each year about 20% of the wheat would be available for food otherwise lost due to diseases (Fakir, 1999; Norhayati *et al.*, 2016). Seed health plays a vital role in the successful cultivation and exploration of yield of crop species. Among various factors that affect the health of seed, the most important are the seed associated fungal pathogens that not only lower the seed germination but also reduces seed vigor resulting in low yield (Huynh *et al.*, 2001). Fungal pathogens are economically most important as they affect seed quality. It is challenging to control fungal seed-borne diseases, as fungal hyphae get established into seed and become dormant. Seed robustness is reduced which results in retarded plant growth at initial stages. Loose smut (*Ustilago tritici*), bakanae disease of rice *(Fusarium moniliforme*), flag smut (*Urocystis tritici*), karnal bunt (*Nevossia indica*) and ear cockle of wheat *(Anguina tritici*) are seed borne diseases (Javaid and Anjum, 2006). Untreated seeds grown in the field are not only responsible for causing variation in plant morphology but also reduction in crop yield up to 15–90% (Wise, 1984). To increase wheat and rice yield, it is obligatory to espouse and practice cheap, eco-friendly and environmentally safe control methods to lessen the incidence of seed borne pathogens. Excessive and persistent application of synthetic antifungal agents exerts adverse effects on environment and may lead to resistance of pathogens against these agents. Varma and Dubey (1999) suggested plant based pest controlling chemicals as better choice to reduce or eliminate the fungal infection associated with seeds as they have minimal impact on environment as compared with synthetic pesticides.

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Lemongrass (*Cymbopogon citratus*) is an aromatic tall herbaceous plant belonging to genus *Cymbopogan* of family *Poaceae* (Akhila, 2010). Lemongrass essential oil possesses biologically active constituents including citral contents comprising more than 75% (w/w) of its essential oil (Huynh *et al.*, 2012; Tajidin *et al.*, 2012). Use of plant extracts and essential oils for the control of seed associated fungi will give an environmental and an eco-friendly solution to control plant pathogens. Moreover there will be low chances of resistance developing in pathogens. Different medicinal plants have been explored for their antifungal potential but no report is available till date for antifungal potential of lemongrass essential oil against seed associated fungi of wheat and rice. Present study was designed to explore the antifungal potential of lemongrass essential oil against seed associated fungi isolated and identified from wheat and rice seeds.

Materials and Methods

Wheat and rice seeds (100 accessions each) were acquired from the Genebank, Plant Genetic Resources Institute (PGRI), National Agricultural Research Centre (NARC), Islamabad (List of accessions is given Table 1 and Table 2). The standard blotter test was used for the isolation of fungi associated with wheat and rice seeds (Doyer, 1938). After seven days of incubation seeds were observed for the presence of associated fungi (Anon, 1996). Number of infected seeds was counted and expressed in percentages as follow:

$$\text{Frequency of occurrence (\%)} = \frac{\text{No. of seeds on which fungal species occur} \times 100}{\text{Total number of seeds}}$$

Pure cultures were obtained after repeated sub-culturing of isolated fungi on potato dextrose agar (PDA) media and fungi were identified on the basis of spore morphology and colony characterization using stereoscopic-binocular microscope (Barnett and Hunter, 1998).

Fresh leaves of lemon grass (*Cymbopogon citratus*) were collected from the clonal repository of medicinal plants of PGRI. Leaves were washed with tap water to remove dirt and dried in glass house at 30±2ºC for 72 h. Dried leaves were packed in plastic bags and stored at room temperature in dark prior to essential oil extraction. Essential oil was extracted from dried lemongrass leaves through hydro-distillation using Clevenger type apparatus. The collected essential oil was dried (over anhydrous sodium sulphate) and stored at 4°C for further use in antifungal activity trials.

Minimum inhibitory concentration (MIC) for lemongrass essential oil against each isolated fungus was determined through agar plug method (Prasad *et al.*, 2010a). Serial dilutions (5–65 ppm) of lemongrass essential oil were added to 20 mL of media (PDA) preceding solidification. One disc (10 mm in diameter) of mycilial plug was taken from seven days old fungal cultures and was transferred to the PDA plates containing lemongrass essential oil. The plates were incubated at alternate periods of dark and light at 28±2ºC for seven days. MIC was determined by measuring the fungal colony diameter.

Disk diffusion method was applied to determine the antifungal activity of different concentrations of lemongrass essential oil (Bansod and Rai, 2008). Fungal spores were harvested from seven days old cultures (isolated from wheat

Table 1: List of rice accessions used in the study

S. No	Acc. No	S. No	Acc. No	S. No	Acc. No	S. No	Acc. No
1	7146	26	7483	51	7542	76	7848
2	7147	27	7488	52	7543	77	7850
3	7148	28	7489	53	7544	78	7851
4	7149	29	7490	54	7546	79	7852
5	7153	30	7493	55	7547	80	7853
6	7164	31	7494	56	7549	81	7854
7	7177	32	7496	57	7553	82	7855
8	7178	33	7498	58	7554	83	7856
9	7181	34	7500	59	7556	84	7857
10	7182	35	7502	60	7557	85	7858
11	7183	36	7503	61	7558	86	7861
12	7184	37	7504	62	7559	87	7864
13	7186	38	7505	63	7566	88	7865
14	7189	39	7509	64	7567	89	7866
15	7190	40	7510	65	7570	90	7867
16	7191	41	7514	66	7575	91	7869
17	7193	42	7516	67	7576	92	7870
18	7196	43	7522	68	7577	93	7871
19	7215	44	7523	69	7580	94	7872
20	7363	45	7525	70	7581	95	7873
21	7401	46	7528	71	7582	96	7874
22	7476	47	7530	72	7585	97	7875
23	7477	48	7534	73	7587	98	7876
24	7478	49	7537	74	7588	99	7877
25	7480	50	7540	75	7847	100	7878

Table 2: List of wheat accessions used in the study

S. No	Acc. No	S. No	Acc. No	S. No	Acc. No	S. No	Acc. No
1	10741	26	11176	51	11211	76	11248
2	10742	27	11177	52	11213	77	11249
3	10748	28	11178	53	11214	78	11251
4	10749	29	11179	54	11216	79	11252
5	11145	30	11181	55	11218	80	11253
6	11146	31	11183	56	11219	81	11254
7	11150	32	11184	57	11220	82	11255
8	11153	33	11185	58	11221	83	11256
9	11154	34	11186	59	11222	84	11257
10	11156	35	11187	60	11223	85	11260
11	11157	36	11188	61	11224	86	11261
12	11158	37	11189	62	11225	87	11262
13	11160	38	11190	63	11226	88	11263
14	11161	39	11192	64	11227	89	11265
15	11162	40	11193	65	11229	90	11267
16	11163	41	11194	66	11230	91	11268
17	11164	42	11195	67	11231	92	11761
18	11166	43	11196	68	11238	93	11782
19	11167	44	11197	69	11239	94	18668
20	11169	45	11199	70	11242	95	010715
21	11171	46	11200	71	11243	96	010717
22	11172	47	11203	72	11244	97	010718
23	11173	48	11207	73	11245	98	010719
24	11174	49	11209	74	11246	99	010720
25	11175	50	11210	75	11247	100	010724

and rice seeds) maintained on PDA. Spores suspension (10^5–10^6/mL) was prepared in sterilized distilled water using haemocytometer. Spores were spread on PDA plates with the help of spreader. Sterilized discs of 5mm diameter (Whatman filter paper No. 1) were immersed in four different concentrations (25 ppm, 50 ppm, 75 ppm and 100 ppm) of lemongrass essential oil prepared in dimethyl solfoxide (DMSO). Discs were dried and placed on bioassay plates using sterile forceps. Fluconazole was used as positive control while, discs impregnated in DMSO only served as negative control. Zones of inhibition (mm) were measured after 7 days of incubation at 28±2ºC.

Each experiment was conducted in triplicate. Data were averaged and means were subjected to analysis of variance (ANOVA) using Statistix 8.1 software. Pair wise comparisons between means were calculated using Duncan's Multiple Range Test (DMRT) at 5% probability level.

Results

Five fungal species *viz*., *A. alternata*, *A. flavus*, *A. fumigatus*, *A. niger* and *D. indica* were isolated from seeds of 100 wheat accessions through Blotter method. Percentage of infection varied from 10–50% in all the accessions from which fungal species were isolated. Maximum fungal infection (50%) was observed in accessions no 11189 and 11242. Two accessions (11172 and 11194) showed 40% infection. Seven accessions (11160, 11164, 11199, 11222, 11239, 11257 and 11261) showed 30% infection. No seed associated fungus was isolated from 64 accessions. Results for the frequency of occurrence of fungal species on wheat seeds are presented in Fig. 1. It is depicted from the results that occurrence of *A. flavus* was the highest (32.87%) followed by *A. niger* (31.50%). Prevalence of both *A. alternata* and *A. fumigatus* was found to be 12.32%. Incidence of *D. indica* was minimum *i.e*., 10.95%.

Total seven fungal species were isolated from rice seeds including *A. alternata*, *A. flavus*, *A. niger*, *C. lunata*, *F. moniliforme*, *D. indica* and *D. tripogonis*. Infection percentage varied from 10–100% in the tested accessions. Maximum fungal infection (100%) was observed in accession no 7496 followed by three accessions (7164, 7178 and 7553) which showed 60% infection. Seven accessions (7148, 7177, 7401, 7476, 7494, 7509 and 7847) showed 50% infection. Four accessions (7147, 7153, 7189 and 7547) showed 40% infection while, from forty six (46) rice accessions, no fungal infection was recorded.

A variation was observed in frequency of occurrence of each fungal species in rice seeds (Fig. 2). The most prevalent fungal species isolated was *A. flavus* (41%) followed by *A. niger* (28%), *D. indica* (10%). Rate of occurrence of *D. tripogonis* was minimum (2%) in rice seeds.

Eight fungal species *viz*., *A. alternata*, *A. flavus*, *A. niger*, *A. fumigatus*, *C. lunata*, *D. indica*, *D. tripogonis* and *F. moniliforme* were selected to test the antifungal activity of lemongrass essential oil. Minimum inhibitory concentration of lemongrass essential oil against each fungus was determined through agar plug method. Results for MICs of lemongrass essential oil against isolated fungal species are presented in Table 3, which clearly indicate that lemongrass essential oil was effective against all strains. Highest MIC (55 ppm) was chronicled for *A. flavus*. Growth of *D. indica* was inhibited at lowest concentration of lemongrass essential oil *i.e*., 20 ppm (Fig. 3). MIC for all the other tested fungal strains *viz*., *C. lunata*, *A. niger*, *A. alternata*, *F. moniliforme*, *D. tripogonis* and *A. fumigatus* was recorded to be 30 ppm.

Antifungal potential of lemongrass essential oil was determined through disk diffusion method. Four different concentrations (25, 50, 75 and 100 ppm) were used during the study. Highly significant differences existed for antifungal activity among lemongrass essential oil/antibiotic, concentrations; and different fungal strains. Interactions between lemongrass essential oil/antibiotic

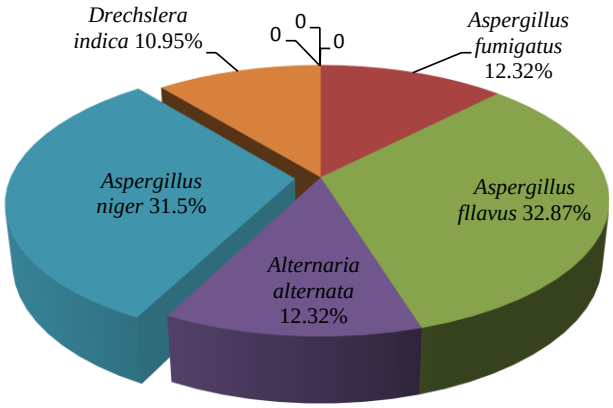

Fig. 1: Frequency of occurrence of seed associated fungi isolated from wheat seeds

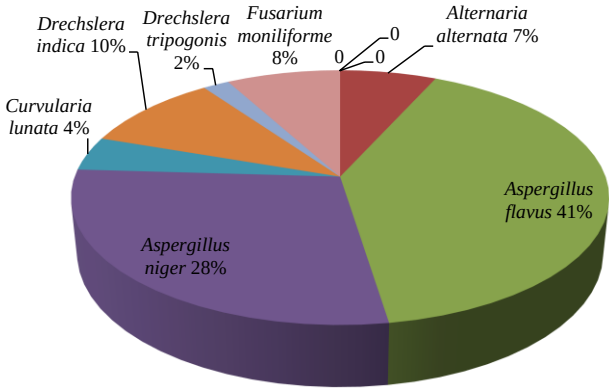

Fig. 2: Frequency of occurrence of seed associated fungi isolated from rice seeds

Table 3: MIC of lemongrass essential oil against selected fungal strains through agar plug method

Fungal specie	Essential oil concentration (ppm)
A. flavus	55
A. niger	30
A. alternata	30
F. moniliforme	30
A. fumigates	30
C. lunata	30
D. tripogonis	30
D. indica	20

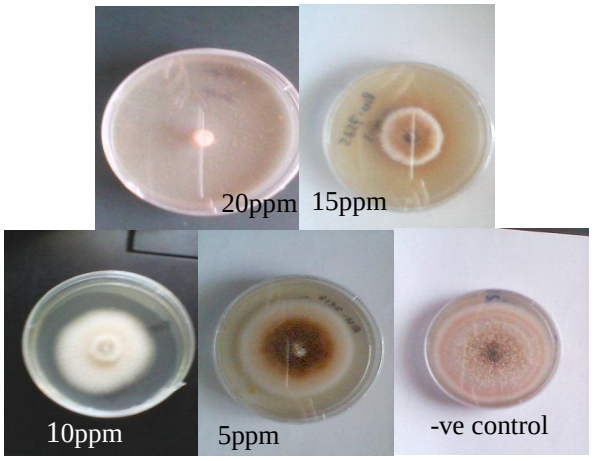

Fig. 3: Effect of lemongrass essential oil on mycilial growth of *Drechslera indica*

and different concentrations; concentrations and different fungal strains were found to be non-significant (P≤0.05). Interaction between different concentrations of lemongrass essential oil and different fungal strains was established as significant (P≤0.05).

Results for consequence of different concentrations on antifungal activity of lemongrass essential oil and antifungal agent (Fluconazole) are presented in Table 4. Highest zone of inhibition (17.75 mm) was recorded for 100 ppm concentration of Flucanazole followed by 75 ppm (17 mm) of the antibiotic. Lemongrass essential oil (100 ppm) inhibited fungal growth by producing a zone of inhibition of 14.0 mm diameter which was comparable to zone of inhibition produced by antifungal agent at 50 ppm concentration. Both fluconzole and lemongrass essential oil showed a concentration dependent antifungal activity. It is clear that 100 ppm concentration of lemongrass essential oil was more effective as compared to lower concentrations.

Antimycotic effect of lemongrass essential oil and fluconazole on isolated fungal strains is presented in Table 5. It is evident from the presented data that *A. niger* was the most susceptible fungus to both Fluconazole and lemongrass essential oil followed by *A. flavus*. The least susceptible species to both lemongrass essential oil and antibiotic was *F. moniliforme* and 10.00 and 11.75 mm zones of inhibition were recorded, respectively.

Table 4: Effect of different concentrations of lemongrass essential oil and antibiotic

Concentrations (ppm)	Zones of Inhibition produced by	
	Lemongrass Essential Oil	Antibiotic
25	9.500^{e}	11.500^{e}
50	11.500^{d}	14.000^{bc}
75	13.250^{c}	17.000^{a}
100	14.000^{b}	17.750^{a}

Mean with same letters are not statistically different at P≤0.05

Table 5: Antifungal activity of lemongrass essential oil and flucanzole against isolated seed associated fungi

Oil/Antibiotic	Fungus	Zone of Inhibition (mm)
Lemongrass E.O	*Fusarium moniliforme*	10.000^{h}
	Drechslera indica	10.000^{h}
	Drechslera tripogonis	10.750^{gh}
	Aspergillus fumigates	11.750^{fgh}
	Curvularia lunata	13.500^{def}
	Alternaria alternata	13.750^{de}
	Aspergillus flavus	14.000^{de}
	Aspergillus niger	14.750^{cd}
Flucanazole	*Fusarium moniliforme*	11.750^{fgh}
	Drechslera tripogonis	12.500^{efg}
	Drechslera indica	13.000^{def}
	Curvularia lunata	14.750^{cd}
	Alternaria alternata	16.000^{bc}
	Aspergillus fumigatus	16.500^{bc}
	Aspergillus flavus	17.500^{ab}
	Aspergillus niger	18.500^{a}

Mean with same letters are not statistically different at P≤0.05

Discussion

Rajput *et al*. (2005) stated fungal infection up to 47.5% in wheat samples collected from Sindh. They identified five seed borne fungal species *viz*., *A. niger, A. tenuis, F. moniliforme, S. herhurum* and *C. lunata*. Fakhrunnisa *et al*., (2006) isolated *Absidia* sp., *Aspergillus sulphureus, F. subglutinans* and *Rhizoctonia solani* from Pakistani wheat varieties. Pathak and Zaidi (2013) accounted the presence of *F. moniliforme, A. alternata, Drechslera* spp. and *C. lunata* as seed associated fungi with wheat seeds. Enikuomehin (2005) recorded higher incidence of *A. tenuis* (17.5%) followed by *C. lunata* (3.7%) in rain fed-areas of Nigeria. This difference in species may be due to difference in ecological zones and wheat samples. A total of 15 different wheat seed borne fungal species were isolated from wheat samples of Central Iran were isolated and identified by Hajihasani *et al.* (2012) with an incidence of 29.1% of *A. niger*. During present study, more or less similar frequency of occurrence of *A. niger* (31.5%) was observed in wheat seeds. Infection rate of seeds depends on environmental conditions like relative humidity, temperature and moisture content in seeds (Niaz and Dawar, 2009).

Ibiam *et al.* (2008) isolated ten fungal species from stored rice grain and six fungal species from rice fields of Ebonyi State. They narrated *F. moniliforme* as most prevalent species both in stored rice grain and rice samples

from field. During present studies, *A. flavus* was found in highest percentage *i.e.*, 41% followed by *A. niger* (28%). Utobo *et al.* (2011) declared the presence of seed borne mycoflora in rice seeds from Nigeria and reported the prevalence of *Trichoconis padwickii, Helminthosporium oryzae and F. moniliforme*. Difference in isolated fungal species could be accounted for difference in ecological zones from where rice seeds were collected. Contrary to present findings, a range of 14 to 27% infection with four fungal species was reported in Basmati rice varieties from Rice Research Institute, Kala Shah Kakoo (Butt *et al.*, 2011). They reported the occurrence of *F. moniliforme*, *Alternaria* sp., *Helminthosporium* sp. and *Curvularia* sp. This may be ascribed due to different rice seed samples used in present study. Other species found to be associated with rice seeds reported by earlier researchers are *P. oryzae, A. padwickii, A. longissina, C. oryzae, D. oryzae, F. semitectum, F. solani, Phoma* sp., *Penicillium* sp., *Myrothecium* sp. and *Collettotrichum* sp. from different rice varieties (Khan, 2000; Wahid *et al.*, 2001; Javaid *et al.*, 2002).

Effect of different chemicals on seed borne fungi have been described by many researchers (Du *et al.*, 2003; Ekefan *et al.*, 2006; Thobunluepop *et al.*, 2008; Butt *et al.*, 2011). During current years, plant based seed treatments are being focused to control seed associated fungi for higher yield. Superfluous application of chemicals antifungal agents is associated with adverse effects like carcinogenicity, environmental pollution and pathogen resistance. As compared to chemical fungicides, plant extracts and essential oils could be a better choice to control and overcome resistance in seed borne mycoflora with slightest upshot on surroundings (Varma and Dubey, 1999).

Essential oils are natural products extracted from various parts of plants and contain a variety of complex mixture of chemical compounds with predominant terpenes associated to alcohols, aldehydes and ketones (Linares *et al.*, 2005; Tzortzakis *et al.*, 2007). Essential oils and plant extracts are being increasingly applied as antimicrobial agents. Several researchers have carried out the antimicrobial studies for medicinal plants including controlling micro-organisms like bacteria, viruses, fungi and insect pests (Singh and Upadhyaya, 1993; Singh, 1996). Cedar wood, clove, citronella, peppermint and nutmeg essential oils have shown significant antifungal potentials against *Phomopsis azadirachtae* (Bouchra *et al.*, 2003; Daferera *et al.*, 2003; Prasad *et al.*, 2010b). Mahesh and Satish (2008) reported the effectiveness of bark and leaf extract of *Acacia nilotica* against *Tinospora cordifolia* and *A. flavus*. Higher concentrations of plant extracts are reported to inhibit mycelia growth (Kocic-Tanackov, 2011). *Cymbopogon martini, Eucalyptus globulus* and *Cinnamomum zylenicum* essential oils have been recounted to possess higher antimycotic activity by Bansod and Rai (2008). *Azadirachta indica*, *Nigella sativa* and *Ferula assafoetida* essential oils were testified against seed borne fungal species by Sitara *et al.* (2008) and *Ferula assafoetida* essential oil was reported to be inhibitory against *A. alternata, A. niger, F. moniliforme, F. oxysporum, F. nivale* and *F. semitectum* at 0.1% concentration.

Lemongrass l is reported to contain higher amounts of essential oil with citral as major constituent (Tajidin *et al.*, 2012). Reports are available regarding antifungal potential of lemongrass and other aromatic plant essential oils against *Candida* spp., and key postharvest pathogens (Tzortzakis et al., 2007; Silva *et al.*, 2008; Tyagi and Malik, 2010). Dose dependent antifungal activity was recorded against different Candida strains for lemongrass essential oil (Silva *et al.*, 2008). Significant variation in antifungal activity of lemongrass essential oil against *C. lunata, A. flavus, A. fumigates, A. alternata* were reported by Mahanta *et al.* (2007). They reported *A. niger* as most sensitive strain against lemongrass essential oil. Present results are in accordance with these findings. Prasad *et al.* (2010b) reported lemongrass essential oil efficacy against *Phomopsis azadirachtae*.

Conclusion

The results of present study prop up the idea that lemongrass essential oil could be used to control seed associated mycoflora of wheat and seeds during storage. Further studies are required to explore antifungal prospective of lemongrass essential oil *in vivo* on stored grain.

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