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ISOLATION, IDENTIFICATION AND CONTROL OF MYCOFLORA ASSOCIATED WITH PADDY

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ABSTRACT

Four varieties of paddy (Super Kernel, Super 515, Super Sindh and Kainaat) were collected from Punjab for the isolation of fungal flora. Eight species with four genera were observed of which, *Penicillium* sp., Link ex. Fr. was isolated and identified on Super Kernel, Super 515 and Kainaat varieties. *Cladosporium* sp., Link ex Gray was observed on Super 515 while *Alternaria alternata* (Fr.) Keissl. on Super Sindh. 1 % Calcium hypochlorite reduced the amount of isolated saprophytic fungi. When paddy seeds treated with microwave radiations and stored at room temperature for 60 days showed reduced carbohydrate and protein contents during storage as compared to control. In case of fungal flora, most fungal species were recorded from Super Kernel while on Kainaat minimum numbers of fungi among all varieties. Surface sterilization with 1% Calcium hypochlorite reduced the incidence of fungal infestation. The fungal species isolated from paddy were *Aspergillus flavus* Link ex Gray., *A. niger* Van Tieghem, *A. ochraceus* Wilhelm, *A. wentii* Wehmer, *Macrophomina phaseolina* (Tassi) Goid., *Penicillium* sp. Link ex. Fr. and *Rhizopus stolonifer* (Ehrenb. Fr.) Vuill.

Key-words: Mycoflora, ISTA technique, microwave treatment, storage at different time interval.

INTRODUCTION

Rice, considered as second largest resource of earning foreign exchange after cotton. It is second staple food after wheat in Pakistan (Nguefack, 2007). Pakistan produced 67,98,000 tones rice in the year 2013-14 covering the area of 27,89,000 hectares with 24,37,000 kg rice per hectare. This way, Pakistan is the 11th largest rice producer in the world and 5th largest exporter of rice. However, the increase in rice production is due to an increase in the cultivated area while the improvement of yield is not up to the mark (Butt *et al.,* 2011). Rice requires diverse range of water content throughout the growing stages. The slow moving water at the level of 15 cm should be given to standing crop. Rice is an important and popular source of complex carbohydrates which fulfills the nutritional needs of two third of the world population. Carbohydrate content of rice grain depends on the activity of various enzymes activity that are linked with carbohydrates synthesis in green leaves and shifted to endosperm (Nakamura *et al.,* 1989).

Quality and quantity of rice are affected by microorganisms, particularly fungi. Many seed and soil borne diseases affect rice growth and these pathogens may establish in areas that were previously unaffected by that particular pathogen like Bakanae disease of rice caused by *Fusarium moniliformae,* is an important example (Neergaard, 1986; Javaid and Anjum, 2006). Storage products may also affect by phytopathogenic fungi which may includes *Aspergillus* spp. and *Penicillium* spp. The affected seeds become discolored, lesser weight than normal unaffected seeds, lesser germinability and produce toxins in them which are in turn harmful for the plant itself, humans, as well as for animals (Neereguard, 1986). In Pakistan, disease causing fungi, includes *Alternaria alternata, A. padwickii, A. longissima, Aspergillus niger, Curvularia oryzae, C. lunata, Drechslera oryzae, Fusarium moniliformae, Pyricularia oryzae* and species of *Phoma, Cercospora, Chaetomium, Sclerotium, Penicillium, Myrothecium* and *Colletotrichum.* These fungi have been isolated from different varieties of rice all over the country (Wahid *et al*., 1993, 2001; Khan *et al.,* 1999; 2000; Javaid *et al*., 2002). Researcher reported that fungi are known to produce 55 diseases in rice that are of great economic importance, among these 43 are known to be seed borne or seed-transmittable (Neergaard, 1979; Richardson, 1979; Leeper 1984; Ou, 1985). Fungi belonging to *Aspergillus* and *Penicillium* develop on rice during their storage after harvesting (Danquah and Mathur, 1976). The germplasm and certain wild varieties are used to promote and improve local varieties but they too are affected by a wide range of fungi like *Alternaria alternata, A. longissima, Aspergillus* spp., *Chaetomium* spp., *Cladosporium* spp., *Cochliobolus lunatus, Colletotrichum* spp., *Curvularia lunata, C. oryzae, C.* spp., *Drechslera oryzae, Epicoccum* spp., *Fusarium moniliformae., F. oxysporum, F. semitectum, Helminthosporium oryzae, Myrothecium* spp., *Penicillium* spp., *Phoma* spp., *Rhizopus* spp., *Rhyncosporium oryzae, Sclerotium* spp., *Trichoderma* spp.,

Trichoconus padwickii and many other fungi as reported by researchers in Pakistan and all around the world (Imolehin, 1983; 1987; Khan *et al.*, 1988; Kim and Lee, 1989; Odebunmi, 1989; Wahid *et al*., 1993, 2001; Khan *et al*., 2000; Javaid *et al*., 2002; Johnson *et al*., 2003; Ibiam *et al*., 2006). The pre and post harvest diseases of rice crop in Pakistan, both in the agricultural field as well as stored form, are responsible to promote the fungal flora in the rice stock (Shanakht *et al.,* 2014).

Contamination of rice stock by mycoflora during storage period depends upon various factors which includes poor aeration in warehouses, high humidity rate, high temperature, mishandling during packaging and transportation at any stage from harvest to consumption by consumer (Shanakht *et al*., 2014). Mycotoxins are secondary metabolites produced by fungi that are toxic for both plant and humans. These mycotoxins produce diverse effects on living tissues depending on their types and concentrations in which they are inhaled or ingested. Toxins which are harmful to humans could be aflatoxins, mostly produced by *Aspergillus* spp. This toxin is carcinogenic, mutagenic and toxigenic (Romagnoli *et al*., 2007; O'Riordan and Wikinson, 2008). *Fusarium* spp., *Penicillium* spp. and other genera are also involved in producing harmful toxins (Roige *et al*., 2009).

Microwave treatment is done for observing different biological, chemical and physiological aspects of rice. Purpose of microwave heating is to improve the physical appearance and chemical properties of food so that the nutritional and sensory properties are retained (Kaasova *et al*., 2001). Basically, microwave treatment can be considered as an alternative to chemically control pathological agents. These do not have unwanted residual effects on the product and is comparatively environment friendly. Moreover it can treat the product within a shorter interval of time thus saving time as compared to other time consuming treatments (Yadav *et al*., 2012). Therefore, main aim of our study was to isolate mycoflora from paddy and control of fungi by using microwave radiations during storage at different intervals.

MATERIALS AND METHODS

Samples collection:

Four varieties (Super Kernel, Super 515, Kainaat and Super Kernel) of paddy were collected from different areas of the Punjab.

Isolation of mycoflora:

Mycoflora was isolated using International Seed Testing Association (ISTA) technique (Anonymous, 1993). It includes three methods that are blotter method, agar plate and deep freezing techniques. Four hundred seeds of each sample of paddy had been tested.

In blotter method untreated and seeds after treatment with 1% Calcium hypochlorite for 5 minutes were placed on three layers of moistened and sterilized blotter paper (20 seeds per Petri dish). Petri plates were incubated for 7 days at 24±1^{o}C under 12h alternating cycles of artificial day light (ADL) and darkness. In case of agar plate method, same method was followed as in blotter method except that Petri plates were poured with sterilized Potato Dextrose Agar (PDA) supplemented with antibiotics (penicillin and streptomycin). Deep freezing method was little bit different in which 1 % calcium hypochlorite treated and non treated seeds were placed on three layers of moistened and sterilized blotter paper were incubated for 24h, each at 20^{o}C and -2^{o}C followed by 5 days incubation at 24±1^{o}C under12h alternating cycles of artificial day light (ADL) and darkness.

In all ISTA suggested methods, mycoflora isolated from paddy seeds after incubation were identified using different literature (Barnett and Hunter, 1998; Domsch *et al*., 1980; Ellis 1971; Raper *et al.*, 1965; Raper and Thom, 1949).

Microwave radiation treatment:

Non sterilized and surface sterilized seeds by 1 % calcium hypochorite for 2-3 minutes were exposed for 10 seconds in a microwave Panasonic (NN-N740 at 120W). Sterilized distilled water (150 mL) was also placed inside microwave for avoiding damage (Reddy *et al.*, 2006). After treatment, seeds were stored for 60 days at room temperature (25-35^{o}C). Blotter method was used for the isolation of fungi.

Moisture content:

Moisture content of paddy was determined by using oven-dry method (ISTA, 2005; Rao *et al.*, 2006) in which 10 g seeds were oven dried at 80^{o}C for 24-48h after which the seeds were weighed again. The experiment was performed in the replicates of thrice.

Carbohydrate and protein analysis

The carbohydrate content of rice was tested using anthrone method as applied by Trevelyan *et al.*, (1952) while protein content was analyzed using Bradford's method (Hassid and Abraham, 1957).

Data analysis

Fungal flora isolated by ISTA technique was analyzed using COSTAT. All means were shown as mean standard error (Gomez and Gomez, 1984).

RESULTS

Identification of mycoflora on paddy: Blotter, agar plate and deep freezing methods were used for detection of seed borne mycoflora of paddy. Eight species with four genera were isolated on four varieties of paddy. Fungi includes *Alternaria alternata, Aspergillus fumigatus, A. flavus, A. ochraceus, A. terreus, A. wentii*, *Cladosporium* sp. and *Penicillium* sp. Super Kernel and Kainaat varieties showed more storage fungi while pathogenic fungi were recorded from Super 515 and Super Sindh ($P<0.05$). Surface sterilization of paddy using 1 % Calcium hypochlorite reduced the number of saprophytic fungi. *Cladosporium* sp., was observed from Super515 while *Alternaria alternata* was isolated from Super Sindh. Saprophytic fungus like *Penicillium* sp., was recorded on Super Kernel, Super 515 and Kainaat varieties (Table 1).

Effect of microwave treatment on mycoflora, moisture content, carbohydrate and protein content during storage: Paddy seeds were treated with microwave radiations and stored for 60 days at room temperature to observe treatment effect on moisture content, mycoflora, protein and carbohydrates contents of paddy. Moisture content was slightly changed in the microwave treated samples of paddy. Results showed that moisture content of paddy decreases with the storage due to reduction of moisture content of paddy during microwave treatment. This is due to the thermal effect that is produced during microwave treatment due to which moisture content was reduced (Wadsworth, 1994). Treated Paddy stored at 15 days showed maximum decrease of moisture as compared to control (Table 2).

The microwave treated varieties of paddy were stored for 60 days and observed for seed borne mycoflora at the intervals of 0, 15, 30, 45 and 60 days by using blotter method as suggested by ISTA (Anon, 1993). Maximum numbers of fungal species were isolated from Super Kernel while Kainaat produced minimum numbers of fungi among all varieties. The incidence of fungal infestation decreased with storage duration in all the varieties but afterwards increased in only Super Kernel with time. This may be due to the fact that air-borne fungal spores get a chance to develop on the seeds. There was no effect on germination of seeds by storage duration (data not shown). The surface disinfection by 1% Calcium hypochlorite reduced the incidence of fungal infestation. The fungal species isolated from paddy were *Aspergillus flavus, A. niger, A. ochraceus, A. wentii, Mcrophomina phaseolina*., *Penicillium* sp. and *Rhizopus stolonifer* (Table 3a,b).

At 0 and after 15 days of storage, minor or no effect in carbohydrate content was recorded in all varieties. However, after 30, 45 and 60 days of storage microwave treated paddy showed decrease in carbohydrate content in Super Sindh, Kainaat and Super 515 (Table 4). All varieties showed decrease protein content in microwave treated paddy. However after 30, 45 and 60 days of storage, Super Sindh, Super 515 and Kainaat gave same level of protein in both non treated and treated with microwave radiation ($P<0.01$; Table 5).

DISCUSSION

Most frequently isolated fungi were *Aspergillus* species which were recorded from all varieties of paddy. These seed borne fungal pathogens cause deterioration of seeds in terms of necrosis, discoloration, deformation and rooting during storage resulting in reduce germination causing impact on yield (Chuku *et al.,* 2010; Nagaraja and Krishnappa, 2009). These saprophytic fungi were somehow reduced due to calcium hypochlorite. Sitara and Akhtar (2007) reported that *Aspergillus niger, A. flavus, A. fumigatus, A. candidus, Rhizopus* spp. and *Nigrospora* spp. were reduced on *Zea mays* seeds due to disinfectant with sodium hypochlorite. It was observed that these chemical disinfectant produce effects on seed biochemistry as these stored seeds are required for future cultivation of crops. These saprophytic fungi produce low molecular weight secondary metabolite known as mycotoxins, producing adverse effect on seed health of crops. Majority of researchers observed toxins of fungi and their relative effect. Most important genera of mycotoxigenic fungi include species of *Aspergillus, Alternaria, Fusarium, Penicillium, Claviceps* and *Stachybotrys. A. flavus, A. parasiticus* produce most potent hepatocarcinogenic substances $B_1(AFB_1)$ (Boudra *et al.,* 2007). Bankole *et al.* (1999) reported aflatoxin B_1 in 32.2% of *Colocynthis citrullus* seeds from Nigeria while Mushtaq *et al.* (2012) detected 21 % of Aflatoxin B_1 in 125 processed food stuff. However Rahim *et*

al. (2016) estimated 32.64, 11.48 and 7.30 ppb, respectively of Aflatoxin B_1 from seed samples of melons using CD-ELISA. Ochratoxin A (OTA) produced by the genera of *Aspergillus* and *Penicillium* responsible to cause gastrointestinal tract problems (Weidenborner, 2001; Kpodo, 1996).

Table 1. Isolation of mycoflora on paddy using ISTA technique.

Name of fungi	Blotter method*		Agar plate		Deep freezing	
	NSs ±SD	Ss ±SD	NSs ±SD	Ss ±SD	NSs ±SD	Ss ±SD
Super Kernel						
Aspergillus wentii Wehmer	10.00±8.0	4.00±1.3	8.00±1.51	3.00±0.54	6.00±1.11	1.00±0.44
Penicillium sp. Link ex. Fr.	4.00±2.8	3.00±0.54	5.00±0.70	4±0.44	1±0.44	0.00±0.00
$LSD_{0.05}$ = Cond.: 4.40; Meth.: 5.39						
Super 515						
Aspergillus flavus Link ex Gray	26.00±3.00	17.00±1.81	21.00±3.27	13.00±1.51	5.00±0.70	2.00±0.54
Cladosporium sp. Link ex Gray.	2.00±0.54	1.00±0.44	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Penicillium sp. Link ex Fr.	1.00±044	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
$LSD_{0.05}$ = Cond.: 7.50; Meth.: 9.19						
Kainaat						
Aspergillus fumigatus Fres.	2.00±0.54	0.00±0.00	1.00±0.44	0.00±0.44	0.00±0.00	0.00±0.00
A. wentii Wehmer	18.00±2.40	14.00±3.11	9.00±3.11	8.00±0.83	5.00±1.00	3.00±0.59
Penicillium sp. Link ex. Fr.	3.00±1.70	3.00±0.44	0.00±0.00	0.00±1.78	0.00±0.00	0.00±0.00
$LSD_{0.05}$ = Cond.: 1.28; Meth.: 1.5						
Super Sindh						
Alternaria alternata (Fr.) Keissl.	9.00±1.64	2.00±0.54	1.00±0.00	1.00±1.64	0.00±0.00	0.00±0.00
Aspergillus ochraceus Wilhelm	0.00±0.00	2.00±0.54	3.00±0.89	7.00±0.44	1.00±0.44	0.00±0.00
A. terreus Thom	6.00±0.44	2.00±0.54	0.00±0.00	1.00±0.4	0.00±0.00	0.00±0.00
$LSD_{0.05}$ = Cond.: 3.7; Meth.: 4.5						

NSs±SD = Non surface sterilized seeds±Standard deviation; Ss±SD = Surface sterilized seeds±Standard deviation; LSD0.05 = Least significant difference at 5% probability level.

Table 2. Effect of microwave treated paddy seeds on moisture content percentage (±SD) stored for different intervals

Time (Days)	0		15		30		45		60	
Varieties	Control	Microwave treated	Control	Microwave treated	Control	Microwave treated	Control	Microwave treated	Control	Microwave treated
Super Kernel	8.7 ± 0.44	8.1 ± 1.31	6.74 ± 0.84	5.7 ± 0.33	7.42 ± 1.09	7.3 ± 2.8	7.21 ± 0.25	8.4 ± 0.9	7.55 ± 1.41	7.16 ± 0.2
Super Sindh	6.63 ± 0.89	6.56 ± 0.83	6.98 ± 0.54	6.37 ± 0.44	6.68 ± 0.89	6.62 ± 0.54	6.63 ± 3.28	6.61 ± 0.89	6.99 ± 1.30	6.54 ± 2.54
Super 515	7.8 ± 0.44	7.36 ± 0.54	7.43 ± 0.62	7.34 ± 0.68	7.77 ± 4.96	7.41 ± 1.51	7.79 ± 0.62	7.68 ± 0.54	7.44 ± 1.09	7.32 ± .64
Kainaat	7.24 ± 1.73	7.21 ± 1.44	7.28 ± 0.89	7.24 ± 2.68	7.31 ± 1.09	7.23 ± 0.89	7.3 ± 0.99	7.25 ± 0.54	7.32 ± 0.83	7.28 ± 0.54
$LSD_{0.05}$	Days = 1.932; varieties = 1.932									

Table 3 a. Effect of microwave treated sterilized (using 1 % Calcium hypochlorite) paddy seeds varieties on microbial infestation stored for different intervals.

Time (Days)	0		15		30		45		60	
Name of Fungi	Control	Microwave treated	Control	Microwave treated	Control	Microwave treated	Control	Microwave treated	Control	Microwave treated
Super Kernel										
Aspergillus flavus	-	-	-	-	-	-	-	-	3±1.14	2±0.44
A. niger	2±0.44	2±0.54	-	-	5±0.44	0±0	-	-	-	-
Penicillium sp.	-	-	-	-	-	-	-	-	-	-
Rhizopus stolonifer	-	-	8±1.34	0±0	-	-	1±0.44	0±0	-	-
Super Sindh										
Aspergillus ochraceus	-	-	-	2±0.55	-	-	-	-	-	-
Macrophomina phaseolina		-	-	-	-	-	6±2.46	-	-	-
Super 515										
Penicillium sp.	-	-	-	-	-	-	4±3.85	-	-	-
Aspergillus wentii	1±0.29	-	-	-	3±1.68	-	-	-	-	-
Aspergillus flavus	-	-	-	1±0.21	-	-	-	-	-	-
Kainaat										
Aspergillus wentii	-	-	8±3.55	-	-	1±0.86	3±4.21	-	-	-

Table 3 b. Effect of microwave treated non-sterilized paddy seeds varieties on microbial infestation stored for different intervals.

Time (Days)	0		15		30		45		60	
Name of Fungi	Control	Microwave treated	Control	Microwave treated	Control	Microwave treated	Control	Microwave treated	Control	Microwave treated
Super Kernel										
Aspergillus flavus	-	-	-	-	-	-	-	-	5±2.8	11±2.30
A. niger	5±0.50	3±0.54	-	-	-	-	-	-	-	-
Penicillium sp.	-	-	-	-	6±1.36	0±0	-	-	-	-
Rhizopus stolonifer	-	-	3±0.54	0±0	-	-	4±3.19	0±0	-	-
Super Sindh										
Aspergillus ochraceus	-	-	-	6±2.65	2±4.72	-	-	-	-	-
Macrophomina phaseolina	-	-	-	-	-	-	2±1.94	-	-	-
Super 515										
Penicillium sp.	-	-	-	-	-	-	5±4.33	-	-	-
Aspergillus wentii	8±0.55	-	-	-	8±3.15	-	-	-	-	-
Aspergillus flavus	-	-	-	-	-	-	-	-	-	-
Kainaat										
Aspergillus wentii	-	-	3±4.85	-	-	-	-	-	-	-

Table 4. Effect of microwave treated paddy on carbohydrate content during different storage periods.

Time (Days)	**0**		**15**		**30**		**45**		**60**	
Paddy varieties	Control	Microwave treated	Control	Microwave treated	Control	Microwave treated	Control	Microwave treated	Control	Microwave treated
Super Kernel	0.78±1.09	0.76±0.89	0.77±0.83	0.77±0.83	0.77±0.54	0.77±0.54	0.78±0.62	0.76±0.89	0.78±1.09	0.77±1.54
Super Sindh	0.76±2.54	0.76±1.09	0.78±8.84	0.77±0.54	0.76±0.44	0.76±1.09	0.79±0.62	0.77±0.44	0.81±0.54	0.81±0.54
Super 515	0.77±1.92	0.71±2.48	0.78±0.54	0.74+4.96	0.77±0.44	0.74±1.34	0.79±0.89	0.74±0.54	0.8±0.83	0.76±0.89
Kainaat	0.77±1.09	0.71±1.51	0.77±0.62	0.77±0.62	0.77±1.30	0.73±2.30	0.78±0.84	0.74±0.62	0.79±0	0.78±0.62
$LSD_{0.05}$	Days = 0.0177; varieties = 0.0177									

Table 5. Effect of microwave treated paddy seeds on protein content during different storage periods.

Time (Days)	**0**		**15**		**30**		**45**		**60**	
Paddy varieties	Control	Microwave treated	Control	Microwave treated	Control	Microwave treated	Control	Microwave treated	Control	Microwave treated
Super Kernel	0.076±1.51	0.071±0.64	0.077±0.89	0.075±0.54	0.077±1.36	0.076±1.36	0.076±0.83	0.075±0.54	0.076±0.44	0.075±0.76
Super Sindh	0.075±1.30	0.075±0.44	0.075±0.84	0.071±1.09	0.07±0	0.07±0.44	0.069±1.09	0.069±0.54	0.071±1.34	0.069±1.72
Super 515	0.069±1.09	0.067±0.54	0.07±2.68	0.069+0.54	0.071±0.83	0.07±2.54	0.068±0	0.068±0.99	0.07±0.54	0.069±0.54
Kainaat	0.074+1.92	0.071±0.49	0.073±1.34	0.07±1.64	0.071±2.48	0.07±0.54	0.071±1.72	0.069±0.83	0.071±0.84	0.07±0.44
$LSD_{0.05}$	Days = 0.0939; varieties = 0.0939									

Many researchers worked on the rice sample to observe the moisture content present in it. Shanakht *et al.* (2014) reported that using five varieties of rice having highest moisture level for a longer duration favors fungal flora as well as mycotoxins. Microwave treatment enhanced the water absorption capacity and starch gelatinization which gave breakage in dormancy during the treatment (Kasova *et al*., 2001). Microwaves effect on seeds was evaluated by several scientists who reported that these radiations eliminated most of the mycoflora (Hari *et al*., 1992). They isolated *Aspergillus candidus*, *Aspergillus niger*, *Eurotium* spp. and *Penicillium* spp. prior to microwave treatment but after the treatment, no fungi appeared. Lozano *et al*. (1986) conducted an experiment to observe the eradication of seed borne mycoflora of cassava seeds. They found that temperature was the most important factor that can control mycoflora, in relation with microwaves treatment and an exposure to microwaves at 77^{o}C is considered as optimum for mycoflora elimination.

Kirkpatrick *et al*., (1972) put forward the report that microwave treatment a high temperature gave 100% mortality rate. Tilton and Vardell (1982) studied the effect of microwaves on seed pathogens and found that low rate of microwave treatment shows low level of pathogens control. Microwave radiations affected carbohydrate content as it was related with the damage of starch content of rice grain and also the power output of the microwave oven that also induces changes in the rice content (Pinkrova *et al*., 2003). Zhao *et al*. (2007) reported that the protein content was decreased after microwave treatment and this decrease was rapid during early storage periods and then gradually slow down with time. Treatment with microwave does not have a notable effect on major nutrient components of rice (Ramezanzadeh *et al.,* 2000).

From the recorded data, it will be suggested that microwave should be considered as effective surface disinfectant for paddy seeds for its protection against or reduction of seed borne mycoflora and maintained their viability. However, further research on this aspect is still needed for the control of other seed borne fungi and long term storage.

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