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Full Length Research Paper

Study of most prevalent wheat seed-borne mycoflora and its effect on seed nutritional value

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To study most prevalent wheat seed-borne mycoflora and its effect on the nutritional value, freshly harvested three and six months old stored wheat grains were collected from various parts of Pakistan. For isolation of seed-borne fungi, agar plate method was found more effective as compared to standard blotter method. *Alternaria alternata* was found to be the most prevalent fungus. Chemical analysis of seeds clearly showed a decrease in the carbohydrate, fats and ash contents of stored wheat grains as compared to the freshly harvested seeds with no effect on total proteins. The growth of *A. alternata* on seeds during storage might have resulted in low nutritional contents.

Key words: Mycoflora, *Alternaria alternata*, seed-borne fungi, post-harvest storage, pathogens.

INTRODUCTION

Wheat (*Triticum aestivum* L.), family Poaceae (Cope, 1984), is a worldwide cultivated grass. Global wheat production is concentrated in Australia, Canada, China, European Union, India, Pakistan, Russia, Turkey, Ukraine and the United States, accounting for over 80% of world wheat production (FAO, 2003). Pakistan is the eighth largest wheat producer, contributing about 3.17% of the world wheat production from 3.72% of the wheat growing area (Shuaib et al., 2007) spanning over 8549.8 thousand hectares in 2007 to 2008 (Agricultural Statistics of Pakistan, 2007, 2008). In Pakistan, a number of different wheat varieties are grown based on varying agro-climatic range. Wheat bears a key position in Pakistan's economy as well as meeting the major dietary requirements of the people of Pakistan as a major cereal crop (Baloch and Irshad, 1986). Wheat cereal is not only a rich source of carbohydrates and proteins but also contains a good blend of minerals (P, Mg, Fe, Cu and Zn) and vitamins like thiamine, riboflavin, niacin and vitamin E (Adsule and Kadam, 1986). However, the content and quality differences among nutrients do exist based on a wheat variety and climatic conditions used for its storage (Chowdhry et al., 1995). In general, different varieties contain 9.15~10.27% of protein, 2.15~2.55% total fats, 1.72~1.85% dietary fibers, 1.44~2.10% ash and 8.38~9.67% moisture content (Khan and Alam, 2007). Seed germination, moisture content, nutritional value, seed discolouration and seed-borne pathogen prevalence have long been known to be influenced by various factors during wheat seed storage.

Stored seeds are regarded as vehicle for plant pathogens over long distances (Agarwal and Sinclair, 1996). Most prevalent pathogens attacking wheat are fungi probably ranked only second to insects as cause of seed deterioration (Christensen and Kaufmann, 1965; D'Mello et al., 1993) and make the wheat grains unacceptable as food and feed (PARC, 1989). Approximately 10 to 15 species of *Aspergillus, Penicillium*, *Fusarium* and *Alternaria* have been reported as important contaminants of cereal grains (Kroiakova et al., 1989; Adisa, 1994; Weidenboner et al., 1996; Klyszejko et al., 2005). These field fungi invade seeds while they are developing on the plants, or in the field and/ after their harvest, and remain alive for years in grains stored at low moisture contents and temperature.

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However, humid and hot climatic conditions favour rapid fungal growth which adversely affects the quality of grain through increase in fat acidity, reduction in germination, mustiness and finally spoilage of grain (Christensen and Kaufmann, 1965). In addition rapidly growing fungi also release a variety of fungal toxins on cereal grains causing health hazards in both human beings and animals upon consumption of contaminated seeds (Hiscocks, 1965). Therefore maintenance of proper wheat seed storage conditions is a pre-requisite to only save huge crop losses from economical view point but to maintain its nutritional quality and protect it from toxic health hazards.

In Pakistan major losses to stored wheat, both quantitative and qualitative, occur mainly because of high temperature and humid climatic conditions during and following harvest season followed by improper storage facilities. Although insects also damage a large quantity of seeds during storage, major losses are due to fungi. Therefore the present research work was designed to compare the mycoflora and to identify the most prevalent fungus on freshly harvested wheat grains and during three and six months storage period. The seeds were also assessed for their nutritional value (Inqalab-91) when freshly harvested and during storage conditions.

MATERIALS AND METHODS

The research work was divided into two phases; isolation and identification of the most prevalent fungus on fresh and stored wheat grains over a period of 3 and 6 months and to test the nutritional contents of wheat grains that is, protein, fats, carbohydrates moisture and ash contents. A total of sixty-three wheat grain samples (Inqalab-91 variety) were collected from Rawalpindi, Attock and Chakwal (seven samples per selected area) in three batches; immediately after harvest and from wheat stores (homes) after an interval of three and six months storage. Seeds were surface sterilized (1.0% NaOCl dip for 1 min and twice rinse with distilled water) and held at 0°C for 92 h to kill any existing mites. Meteorological data of the area under study like; mean temperature, mean relative humidity and total rainfall, was also collected from Pakistan Meteorological Department, Islamabad on monthly basis. The moisture content of all wheat grain samples was determined by drying 10 g seeds over night at 105°C in hot air circulating oven (AACC, 1962).

For fungal growth and isolation, seeds were incubated on Agar plates (Annonymous, 1976) and seed-borne fungi, in the form of fungal colonies, were identified (Barnett and Hunter, 1972; Booth 1971; Ellis, 1971; Nirenberg, 1976; Nelson et al., 1983; Raper and Fennel, 1965) and counted directly under a stereomicroscope. In case of multiple fungal growths, colonies were isolated from a single wheat grain, recorded and pure cultures were maintained on PDA slants (pH 5.6). The fungal colonies were recorded in terms of percentage frequency (Fr) of species and calculated as follows:

$$\text{Percent frequency (\%)} = \frac{(ns)}{N} \times 100$$

Where ns represents the number of samples, where a genus or species occurred; N indicates the total number of samples tested. The most prevalent fungus was maintained in sporulation medium (Fapohunda, 1992). All sterilizations before inoculation were done at 121°C for 15 min. For *in vitro* inoculation of fungus, 20 g of ground wheat sample (80 mesh) were placed in a flask and mixed with 50 ml distilled water added to make a suspension. The fungus was cultivated on nutrient broth for seven days, culture filtered and diluted to give 3.5×10^6 conidia/ml (Stephen et al., 2006). These asexual spores served as inoculum for the blended grains and incubations were carried out at 20, 25, 30, 35 and 40°C for 5 and 10 days. The biochemical analysis are expressed as percentage ash, crude protein, crude fat, total carbohydrate and moisture contents were determined using dry samples (AOAC methods Helrich, 1990). The uninoculated flasks were similarly treated and served as a control.

RESULTS AND DISCUSSION

To isolate and identify the most prevalent wheat born seed fungus and its effect on nutritional status, a total of 63 wheat samples, comprising of 42 stored and 21 freshly harvested samples freshly harvested as well as 3 and 6 months old stored wheat grains were subjected for analysis. Meteorological data regarding mean temperature, relative humidity and total rainfall of the area under study that is Rawalpindi, Attock and Chakwak were obtained from Pakistan Metrological Department (www.pakmet.com.pk) on monthly basis. The moisture contents of freshly harvested wheat grains were low as compared to 3 and 6 month old stored samples (Table 1). The moisture contents reported in present study are slightly higher as compared to 8.38 to 9.67% reported from Pakistan earlier (Khan and Alam, 2007) especially for wheat varieties from Dera Ismail Khan (Jamil and Khan, 2002). The main reason for lower moisture content in Dera Ismail Khan could have been due to its hot and very dry area due to less rainfall thus decreasing moisture content. The moisture content also showed an increased trend with an increase in the length of seed storage and invasion of storage fungi (Barton, 1961; Harrington, 1963).

Isolation and identification of wheat grain mycoflora

Culture of freshly harvested wheat using agar plate method, eight genera and 13 species were isolated. Along with *Alternaria alternata* other species were; *Alternaria tenussima, Fusarium nivale, Fusarium graminearum, Fusarium heterosporum, Fusarium proliferatum, Fusarium sporotrichioides, Fusarium tricinctum, Fusarium semitecum, Aspergillus niger, Mucor* spp., *Rhizopus* spp., *Curvularia lunata*, *Bipolar specifera* and *Stemphylium herbarum.* Percentage of grain samples yielding fungal samples was also calculated and showed 57, 54 and 46% of *A. alternata* from Rawalpindi, Attock, and Chakwal Districts, respectively (Figures 1, 2 and 3). Similarly, in standard blotter method, *A. alternata* predominated in freshly harvested and three month storage wheat grain samples, followed by *Aspergilllus* and *Rhizopus,* respectively (Figures 4, 5 and 6). The other prominent genra present in fresh as well as in stored wheat grain samples were *Aspergilus, Rhizopus,*

Table 1. Moisture contents of the freshly harvested, three and six month stored wheat grain samples.

Storage period (months)	Moisture (%)*		
	Rawalpindi	Attock	Chakwal
0	10.75±0.70	10.82 ±1.06	9.75 ±0.56
3	13.87±1.18	13.66 ±1.30	12.22 ±0.80
6	14.38±1.04	13.87±0.96	13.09±0.65

*Values are means of three readings with standard deviation.

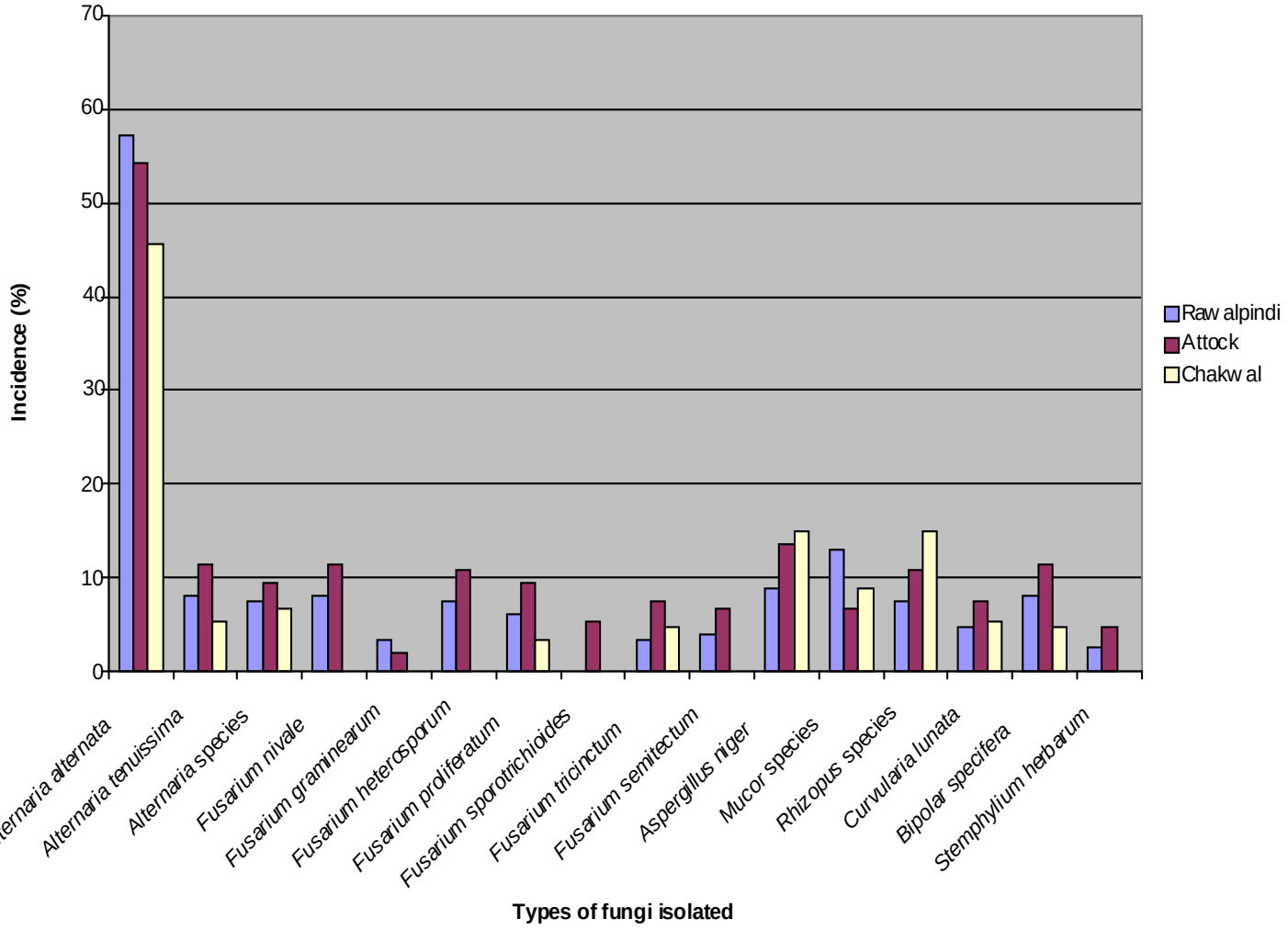

Figure 1. Incidence percent of mycoflora on freshly harvested wheat grains of Districts Rawalpindi, Attock and Chakwal by agar plate method.

Mucor and *Fusarium* (especially in fresh wheat grains). Around six *Fusarium* spp. were isolated from fresh wheat grains in both Rawalpindi and Attock as compared to four in District Chakwal (Figure 4). *Absidia* spp. was isolated only from 3 to 6 months stored wheat grains of all regions except District of Chakwal. In case of six months stored wheat (Figure 6) *A. alternata* was most prevalent in the District of Rawalpindi (20%) and Attock (21%), whereas, in the District of Chakwal, *Aspergilus niger* (24%) and Rhizopus spp. (24%) were found prevalent with only 12% *A. alternata.* The low occurrence of *A. alternata* in Chakwal could have been due to appearance of storage moulds, especially *Penicillium*, a strong *Alternaria* spp. anatagonist (Wallace and Sinha, 1962).

For fungal isolation, agar plate method was found more efficient as more than ten fungal genra were isolated on

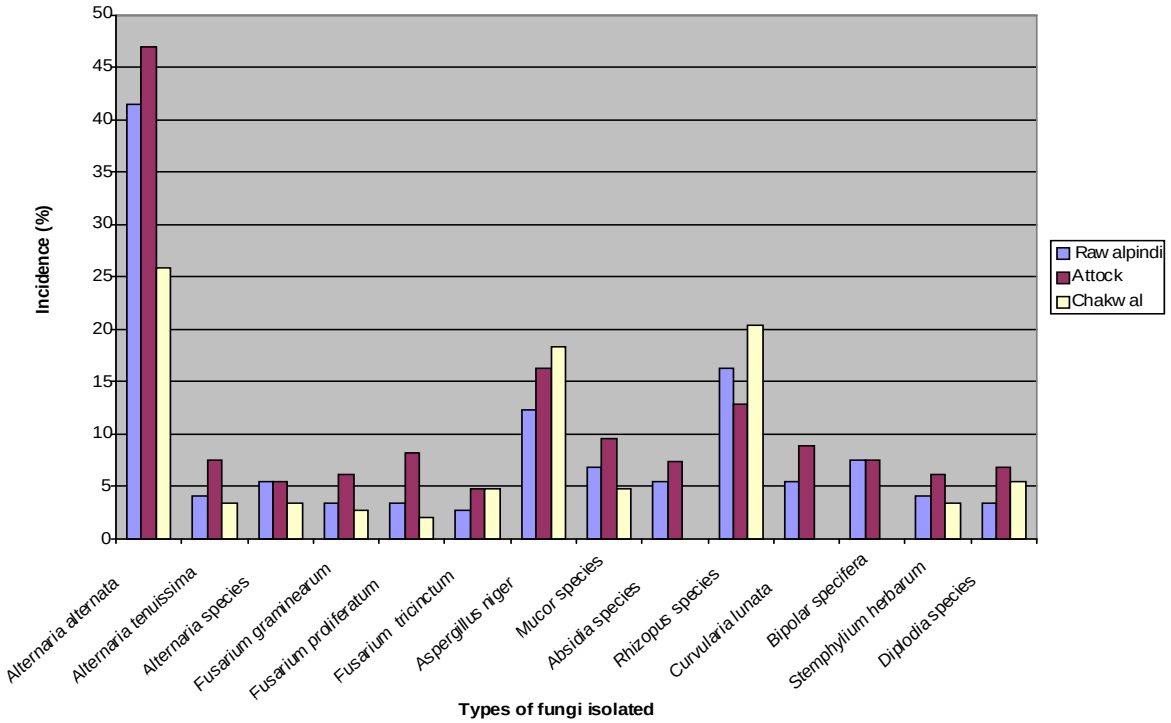

Figure 2. Incidence percent of mycoflora on three month storage wheat grains of Districts Rawalpindi, Attock and Chakwal by agar plate method.

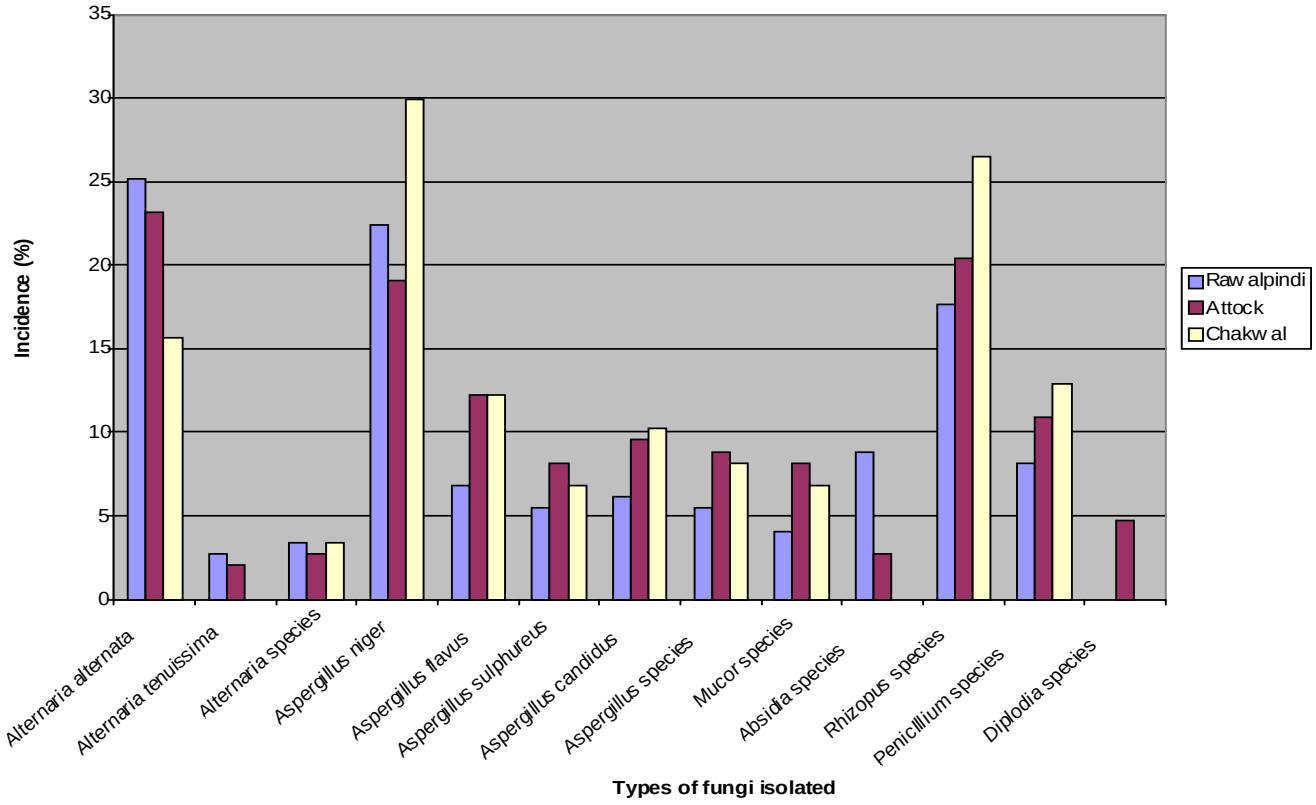

Figure 3. Incidence percent of mycoflora on six month storage wheat grains of Districts Rawalpindi, Attock and Chakwal by agar plate method.

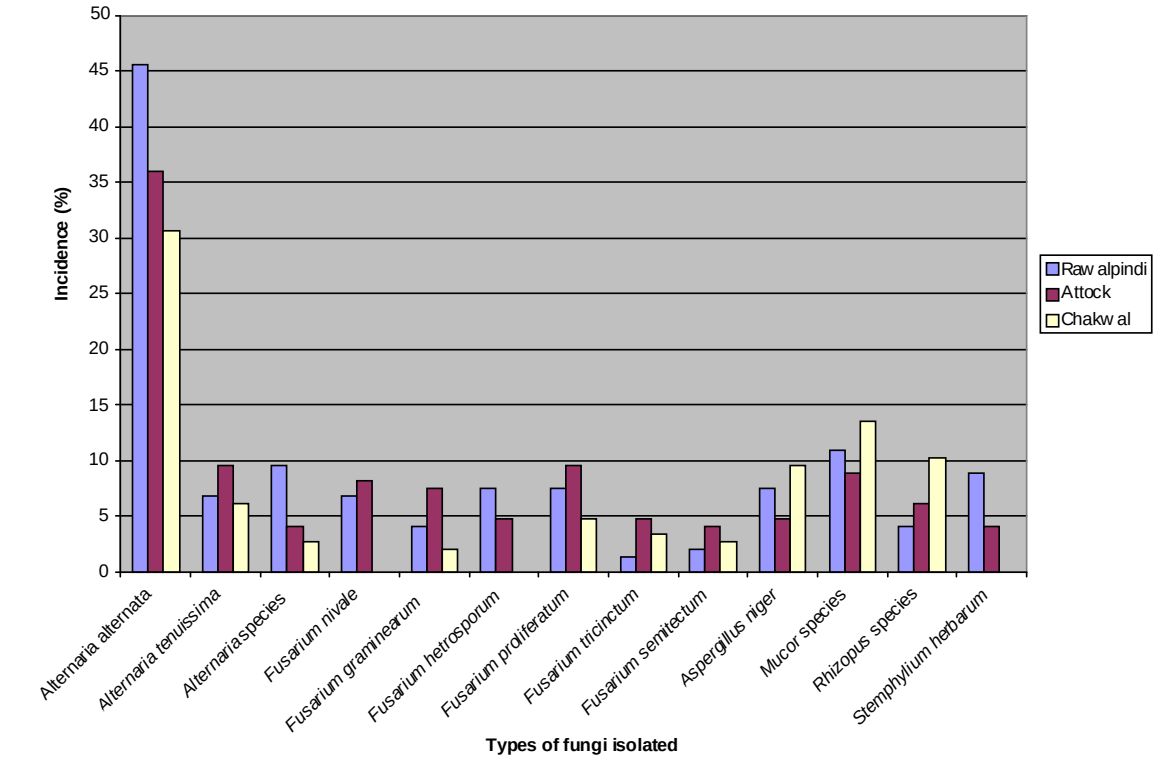

Figure 4. Incidence percent of mycoflora on freshly harvested wheat grains of Districts Rawalpindi, Attock and Chakwal by blotter method.

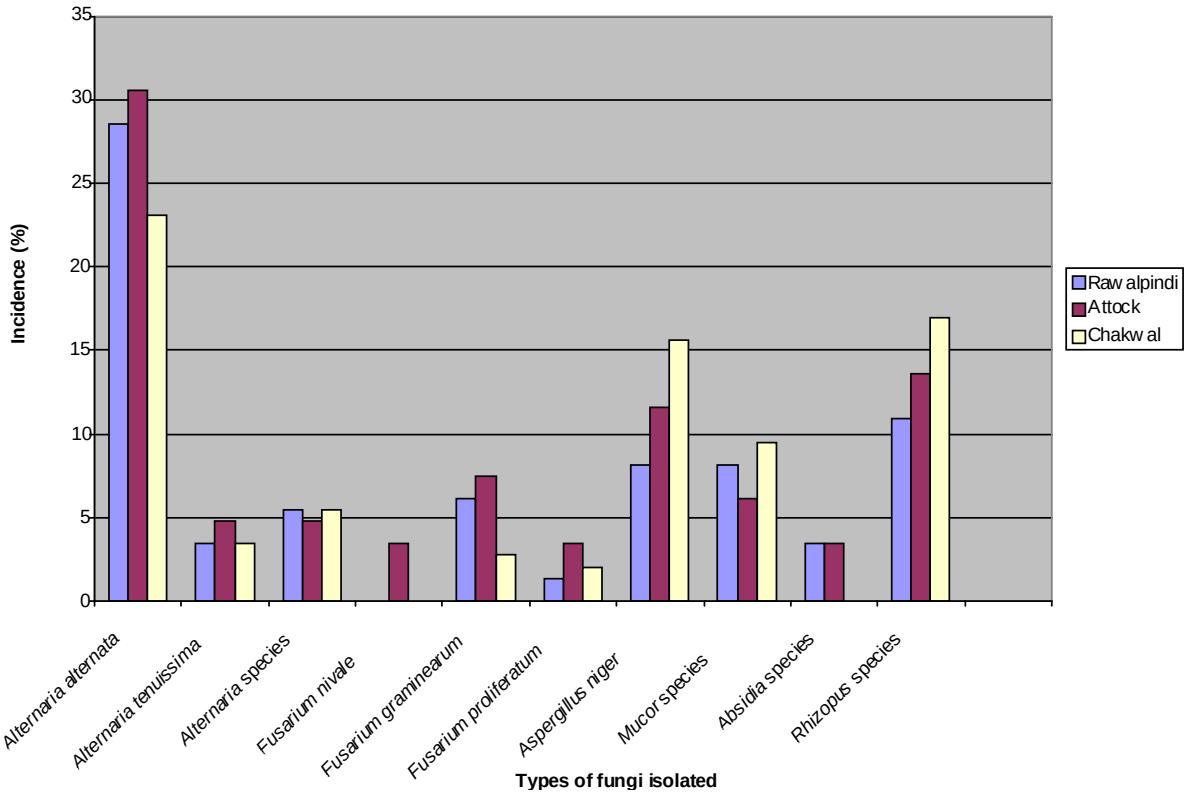

Figure 5. Incidence percent of mycoflora on three month storage wheat grains of Districts Rawalpindi, Attock and Chakwal by blotter method.

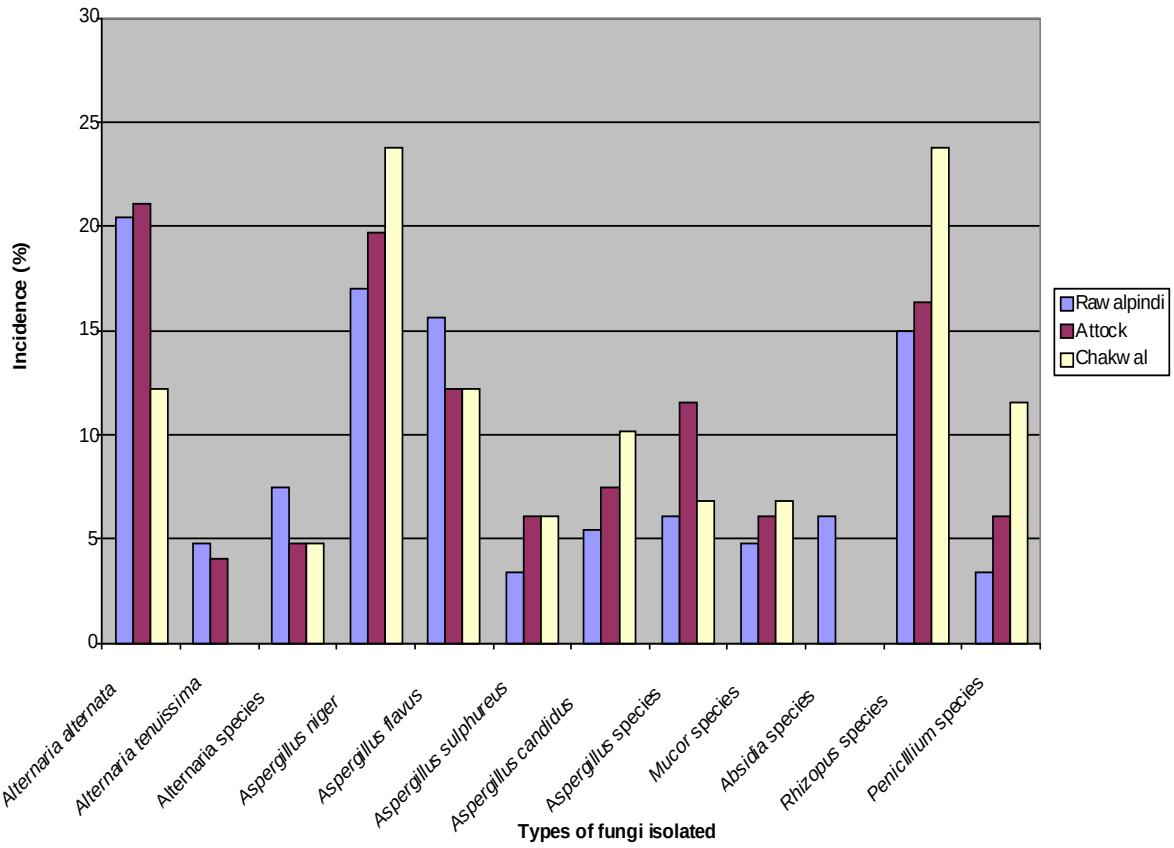

Figure 6. Incidence percent of mycoflora on six month storage wheat grains of Districts Rawalpindi, Attock and Chakwal by blotter method.

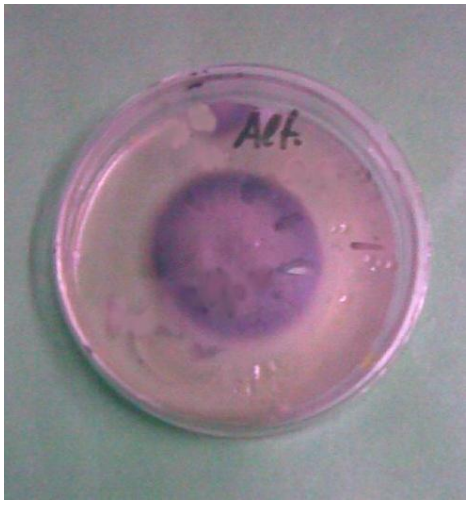

Plate 1. Growth pattern of *A. alternata* on PDA.

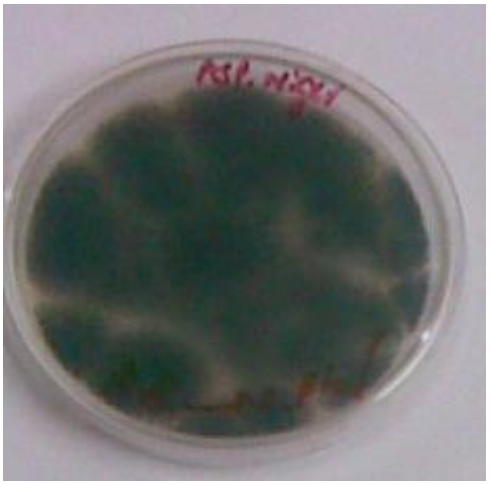

Plate 2. Growth pattern of *A. niger* on PDA medium.

plates as compared to bottler method (Niaz and Dawar, 2009) especially for the detection of *Aspergillus* spp., *Cladosporium* spp., *Curvularia* spp., and *Rhizopus* spp. The isolated fungi were identified on the basis of colony, hyphae, and conidial characteristics on PDA. *A. alternata* colonies were initially green and then turned to blackish colour (Plate 1) with club shaped golden brown conidia having several vertical and horizontal partitions. *A. niger* colonies were dark brown to black in colour (Plate 2), conidia black, globose to sub globose in nature and about

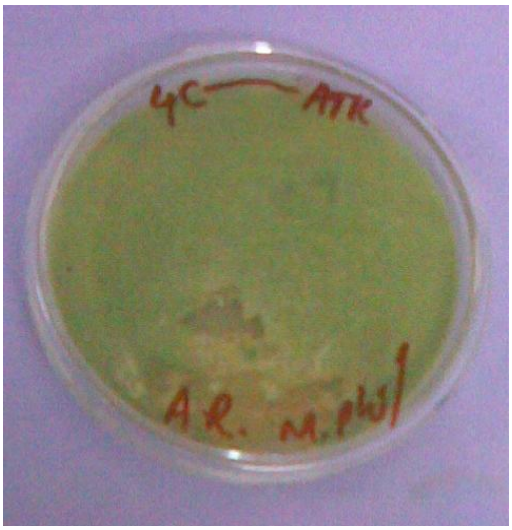

Plate 3. Growth pattern of *A. flavus* on PDA medium.

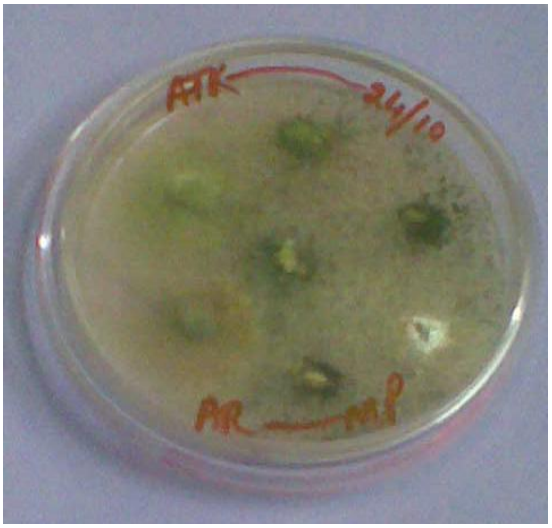

Plate 4. *A. niger, A. flavus, A sulphureus Penicillium.*

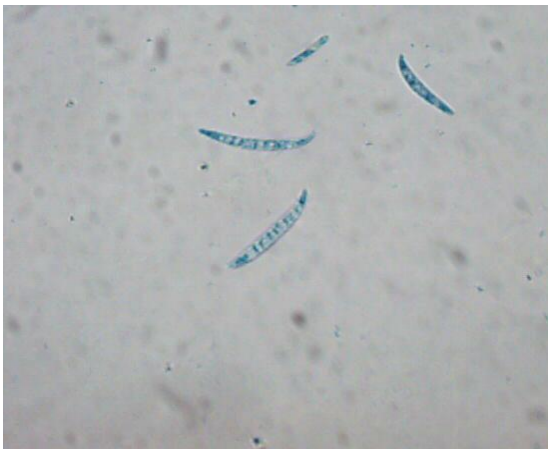

Plate 5. *F. graminareum.*

Plate 6. *F. graminareum* on PDA medium.

5 µm in diameter. Granular colony of *Aspergillus flavus*, initially yellow, turned bright to dark yellow green with age (Plate 3) with globose to sub globose conidia about 6 µm in diameter. *Rhizopus* spp. was characterized by the presence of stolons and rhizoids with fast growing, white cottony colony which turned grey. *F. graminearum colonies* appeared white creamy (Plates 5 and 6) while *Penicillium* spp. conidiophores were branched with brush like structures. *Curvularia lunata* had septate conidia about 20 µm long with fluffy black colour. Mucor spp. was white to yellow in colour, filamentous sporangia 260 µm in diameter, possessing septate stolon and no rhizoids. The graphical data of fresh and stored wheat grains clearly shows that *A. alternata* was the most prevalent fungus in present study. The fungus growth was maintained in sporulation medium (Fapohunda, 1992) and its effects on nutritional value of ground wheat grain was observed.

Effect of *A. alternata* on nutritional qualities of ground wheat grains was carried out by incubating samples at 25 and 30°C as 28°C is the optimum growth temperature for *A. alternata*. The results showed an increase in crude protein and ash contents with a reduction in relative quantities of fat and protein (Fittenborg et al., 1996) in samples incubated at 25 and 30°C as compared to controls (Plates 7 and 8) at the same temperature. The maximum increase in crude protein contents was observed at days five and ten during incubation. The reason for increased protein levels in incubated samples could have been due to the production of single cell proteins by *A. alternata* in accordance with a previous work on single cell protein production by *Penicillium expansum* (Yaqoub et al., 1992).

Finding of present investigation showed a negative

Plate 7. Controlled and affected wheat flour with *A. alternate.*

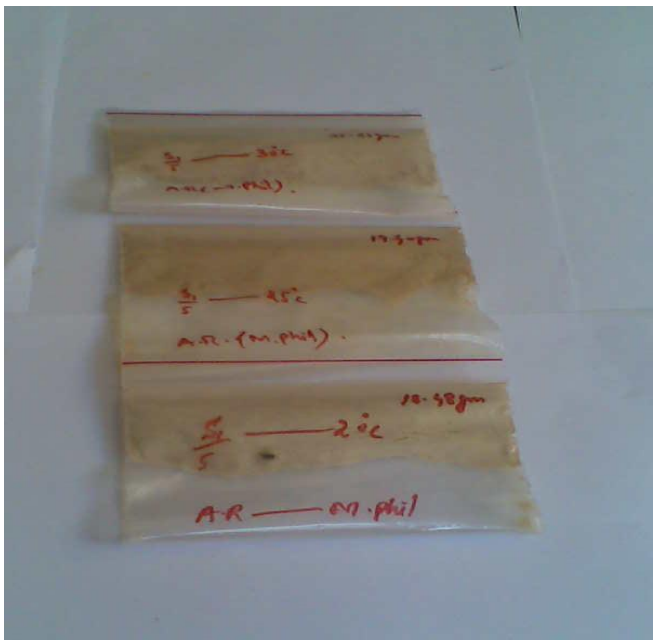

Plate 8. Colour changes in ground wheat grains contaminated with *A. alternate.*

effect of *A. alternata* on the carbohydrate contents of the blended wheat grains decreased to their maximum in samples incubated at 25 and 30°C as compared to controls. Our results are in line with Stephen et al. (2006), who also showed a decrease in fat, fibre and carbohydrate contents of stored and incubated cereals by *Alternaria tenuisima* degradations. However, Stephen et al. (2006) observed a decrease in protein percentage in contrast to an increase in protein percentage reported in present study which could have arised due to some single cell protein production by *A. alternata*. Further studies need to be conducted to confirm single

Table 2. Effect of *A. alternata* on ground wheat grains at different temperatures (after five days of incubation).

Sample	Proximate analysis	Temperature of substrate (°C)									
		Control value	20	Control value	25	Control value	30	Control value	35	Control value	40
Wheat	Moisture %	7.22±0.05	8.11±0.08	7.39±0.04	9.10±.0.08	6.95±0.09	9.36±0.10	6.38±0.05	7.28±0.11	6.49±0.07	7.23±0.15
	Total mineral (ash)	1.44±0.11	1.89±0.09	1.64±0.14	2.39±0.09	1.58±0.15	2.97±0.11	1.37±0.06	1.82±0.08	1.59±0.21	1.89±0.11
	Crude protein (%)	12.25±0.05	13.75±0.11	12.12±0.	14.24±0.14	11.92±0.15	14.67±0.12	11.88±0.08	13.22±0.14	11.78±0.12	12.37±0.11
	Crude fat (%)	2.65±0.05	2.53±0.11	2.62±0.07	2.30±0.09	2.57±0.12	1.95±.0.08	2.59±0.10	2.05±0.06	2.48±0.14	2.33±0.12
	Total carbohydrate (%)	79.1± 0.35	76.45±0.28	78.59±0.27	68.86±0.29	77.40 0.42	65.27±0.57	76.77±0.34	74.86±0.23	76.50±0.28	75.30±0.36

Values are percent means of two readings with standard deviations.

Table 3. Effect of *A. alternata* on blended wheat grains at different temperatures (after ten days of incubation).

Sample	Proximate analysis	Temperature of substrate (°C)									
		Control value	20	Control value	25	Control value	30	Control value	35	Control value	40
Wheat	Moisture (%)	7.21±0.05	8.37±0.08	7.02±0.09	9.57±0.08	7.04±0.11	9.73±0.06	6.46±0.11	8.18±0.04	6.38±0.07	7.39±0.06
	Total mineral (ash)	1.37±0.06	2.02±0.07	1.56±0.09	2.55±0.10	1.49±0.08	3.05±0.11	1.42±06	2.05±0.09	1.48±0.08	1.98±0.05
	Crude protein (%)	12.04±0.07	13.92±0.13	12.05±0.08	14.70±0.12	11.85±0.09	14.84±0.14	11.72±0.06	13.45±0.11	11.67±0.06	12.56±0.09
	Crude fat (%)	2.54±0.07	2.39±0.08	2.57±0.06	1.98±.0.13	2.51±0.05	1.99±0.09	2.39±0.08	2.19±0.09	2.41±0.09	2.26±0.08
	Total carbohydrate (%)	78.36±0.15	75.45±0.24	78.04±0.25	67.30±0.32	76.77±0.17	63.18±0.28	75.68±0.24	73.48±0.33	75.27±0.12	74.85±0.28

Values are percent means of two readings with standard deviations.

cell protein contribution towards total stored seed protein contents for an accurate estimation. The increased moisture content in the incubated samples could have been due to normal response to fungal respiration in an environment of oxygen. The increase in moisture content was observed for all the temperatures for both types of incubated samples as compared to controlled samples, but it was much prominent at 30 and 35°C. The data presented in Table 2 shows optimum temperature for degradation of crude fat at 30 to 35°C for incubation of 5 days while the degradation of crude fat is observed at 25 to 30°C at 10 days incubation (Table 3).

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