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Pathological Evaluation and Quantification of Some Seed-Born Fungi of Wheat

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Five pathogenic fungi (*Alternaria alternata*, *Bipolaris sorokiniana, B. spicifera, Fusarium avenaceum* and *F. graminearum*) were evaluated for seed discoloration, shriveling, germination and seedling vigor of wheat. Three wheat cultivars (Yecora Rojo, Logame and Sammah), two inoculation times (at anthesis and 12 days later) and two environmental conditions (wet and dry) were used in this investigation. Considerable differences were recorded between the tested fungi for seed discoloration, shriveling, germination and seedling vigor at each treatment. Fungal mycelia were also assessed in wheat grains via estimation of chitin and ergosterol contents. For each fungal species, significant differences between samples were detected. It is evident that visual characteristics of seed provide an imperfect guide to the extent that seed is infected by fungi. Chemical analysis for either chitin or ergosterol, if developed for routine use, could well be helpful in identifying potentially samples on which additional analyses for specific mycotoxins could be carried out.

Key words: *Alternaria alternata*, Chitin, Discoloration, Ergosterol, *Bipolaris sorokiniana* , Seed germination and Shriveling.

Wheat is a nutritious, convenient and economical source of food. It provides about 20% of the world's food calories and is a staple for nearly 40% of the world's population. Wheat, like other cereals is subject to attack by many groups of pathogens (fungi, bacteria, nematodes and viruses). The fungi comprise the largest and best studied group of wheat pathogens[1]. Many affect the grain causing injury which detracts from its value either as food or as seed. Wheat grains are almost invariably infected to some extent with fungi. More than 150 species of yeasts and filamentous fungi have been reported on cereals grains[2]. These are normally divided into two groups' viz. field fungi and storage fungi. The former group infects the grain during its development whereas the latter develop mainly after harvest when the grain is in store. Storage fungi may be present in or on wheat seeds before harvest but normally only develop during storage. The major storage fungi are species of *Aspergillus* and *Penicillium* and their development can be restricted by storing grain under cool, dry conditions. Field fungi are more difficult to control; their spores are usually readily airborne and are able to infect grain even in dry climates although they are much more prevalent in seed that develops during warm wet weather[3]. The field fungi which are more frequently associated with discolored kernels are species of *Bipolaris*, *Alternaria*, *Fusarium*, *Cladosporium*, *Nigrospora*, *Penicillium*, and *Stemphylium*[4].

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Some seed-borne fungi have been found to be very destructive, affecting seed germination and early seedlings growth, grain quality (discoloration), grain size and weight[5] and presence of mycotoxins. Both the severity and incidence of infection in infected wheat grains vary greatly. One of the most disturbing and emotive aspects of invasion of grain by fungi is the possible production of powerful mycotoxins which survive to contaminate food products[6]. Several fungi (both field and storage fungi) are now known to produce mycotoxins, the most important being *A. flavus* and species of *Fusarium*[7,8]. Fortunately, with most crops there is a fair correlation between visible mould damage and mycotoxin contamination. Badly contaminated samples are, therefore, unlikely to be processed for human food and are likely to be avoided by domestic animals. Further, animal feeds are normally mixtures of grain samples; mycotoxin levels are thus subject to dilution[9]. Occasionally grains may contain appreciable amounts of mycotoxins but show little or no evidence of fungal infection. In these instances visual symptoms are a poor guide to the extent of fungal content. The use of assays based on specific fungal metabolites (ergosterol or chitin), if they can be developed for routine tests, may prove a better guide to fungal infection and potential, mycotoxin levels.

The problems of fungal infection and discoloration of grain has attracted attention over many years but there are many aspects of the problem which remain unresolved. The studies described in this work have been broad ranging but have focused mainly on methods to quantify fungal infection in wheat grains.

MATERIALS AND METHODS

Seed Samples and Surface Sterilization

Four seed samples were used. Samples 1 and 2 were of a USA cultivar (Yecora Rojo) recently imported into Saudi Arabia; sample 1 was grown in the USA and had been treated with a fungicide whereas sample 2 was seed once-grown in Saudi Arabia which had not been treated with fungicide. Samples 3 and 4 were local Saudi Arabian cultivars (Logame and Sammah respectively) and had not been treated with fungicide.

Isolation, purification and identification of the seed-borne fungi

Sub-samples (50 seeds) were taken at random from each of the four samples and each sub-sample was wrapped in a small muslin bag to facilitate transfer through the sterilant and successive changes of sterile water. Two sterilants were used viz. 10% sodium hypochlorite and 0.1% mercuric chloride. After immersion for 6-7 minutes seeds were washed in six changes of sterile distilled water (5 min in each change). Seeds were plated (10 per plate) on potato dextrose agar ('Lab-M') containing 100 ppm streptomycin sulphate to suppress bacterial growth. Plates were incubated for 7 days at 25°C. At the end of this time the numbers of fungal colonies produced on each plate were recorded. Purification of the isolates was done using the single spore or hyphal tip techniques to obtain them in pure colonies. Pure cultures of the isolated fungi were identified according to their cultural properties, morphological and microscopical characteristics as described by Booth[10]; Domsch *et al*.[11] and Burrges *et al*.[12]. Non-sporulating colonies were sub-cultured and incubated under near ultraviolet light to stimulate sporulation.

Evaluation of some seed-borne fungi on wheat plants

Preparation of inocula

Cultures of five fungi (*A. alternata, B. sorokiniana, B. spicifera, F. avenaceum* and *F. graminearum*) isolated from wheat seeds were used. These were grown on potato dextrose agar (PDA) in Petri plates at 25°C for 14 days. Spores were washed from the plates with sterile distilled water and the suspension filtered through sterile muslin cloth. Spore concentrations were adjusted to 10^6 spores $m1^{-1}$.

Planting and growth conditions

Seeds from each sample were pre-germinated on moist filter paper at 22°C and the seedlings sown in 19 cm pots containing John Innes Compost No. 2. Five plants were sown in each pot. The pots (60 for each seed sample) were arranged in four randomized blocks; within each block pots were placed in three rows, one for each inoculation time, within rows samples and fungal treatments were randomized. The experiment was established in a glasshouse. Compound fertilizer (20, 10, 10, N, P, K) was applied (3.5g per pot).

Three inoculation treatments were examined. The first inoculation (I_1) was made at anthesis (60 days-old); the second (I_2) was made 12 days later and the third inoculation (I_3) was the water sprayed control. The plants, after inoculation, were maintained under wet and dry conditions which were provided in the same pot half of the heads being maintained under the wet and the other half under the dry conditions. For the wet condition, plants were enclosed in plastic bags immediately after inoculation to maintain high humidity. Bags were removed after three days for one day and then covered again for three days, this regime being maintained up to harvest. The dry condition was provided by not covering the plants at any time. Water was always applied to the soil, never to the aerial parts of the plants. Plants were harvested at 90 days -old. The heads were threshed by hand. The weight of 100 seeds from each sample was recorded.

Assessment of discoloration and shriveling

Two sub-samples of 100 seeds were withdrawn at random from each sample of harvested grain. On one sample the number of grains showing blackish or brownish discoloration was determined; on the other sample the number of shriveled grains (whether discolored or not) was determined.

Assessment of seed germination and seedling vigor

Grains from each of the 96 samples were tested for germination and seedling vigor using the rolled paper towel method developed by Warham[13]. The number of germinated seeds was recorded. Seedling vigor was based on shoot length. Mean seedling length was calculated from germinated seeds as total length of germinated seeds/number of germinated seeds.

Isolation of fungi from seeds

Five seeds from each of the 96 samples were surface sterilized in 10% sodium hypochlorite for 7 min and washed in three changes of sterile distilled water before plating on PDA and incubating at 25°C for 7 days. At the end of the incubation period plates were checked for total colonies of the predominant fungal species.

Analysis of data

Measurements of discoloration, shriveling and germination were all determined as percentage values which were angularly transformed before analysis. Measurements of shoot length were transformed to square roots and measurements of seed weight were not transformed.

Assay of chitin and ergosterol

Vogel's Minimal Medium was used. Aliquots (20 ml) were dispensed to 100 ml conical flasks before autoclaving. Each flask was inoculated with a single plug (5 mm) cut from a young pure culture and the flasks incubated in the dark at 25°C. Cultures were harvested at 2, 4, 6, 8 and 10 days for chitin assay and at 2, 6 and 10 days for ergosterol assay. The fungal mycelium was collected on a 50 micromesh sieve, washed with distilled water and homogenized in water (15 or 20 ml) using a 'Quick-fit' homogenizer. Three aliquots (3 ml) of the suspension were transferred to aluminum foil boats and dried to constant weight at 100°C. The remainder of the suspension was used for analysis.

Chitin assay of the mycelial suspensions

Duplicate aliquots of the mycelial suspension (0.9, 0.6 and 0.3 ml) were transferred to 15 ml graduated, tapered glass centrifuge tubes and centrifuged (5000 rpm for 10 min). The residue was assayed for chitin according to the method of Ride and Drysdale[14].

Chitin assay of the wheat grains

Wheat grains (5 g) were ground using a gristmill and the flour kept in bottles until used. Samples of flour (100 mg) were placed in plastic centrifuge tubes (15 ml) and washed with 5 ml acetone, then centrifuged (5000 rpm for 10 min). The residue was assayed for chitin according to the method of Ride and Drysdale[14] as modified by Nandi[15].

Ergosterol assay of the mycelial suspensions

Aliquots (2 ml) of mycelial suspensions were centrifuged at 1000g, the supernatant discarded and the residue extracted with 200 ml methanol by shaking for 1 h in darkness. The extract was then filtered (Whatman No. 5 paper) and assayed for ergosterol according to the method of Molope and Page[16].

Ergosterol assay of the wheat grains

Samples of flour were extracted with 100 ml methanol by shaking for 1 h in darkness. The extract was centrifuged for 10 min at 1000g. The extract was filtered and assayed for ergosterol according to the method of Molope and Page[16].

RESULTS

Isolation of wheat seed-borne fungi

Data presented in Table 1 shows that total number of colonies in samples 2, 3 and 4 were similar but few colonies were recorded from sample 1. Differences between sterilization treatments were small with, overall, more colonies produced following treatment with sodium hypochlorite. A wide range of fungal species was recorded but *A. alternata* was by far the most common accounting for 70% of all colonies. Species of *Bipolaris* (*B. sorokiniana* and *B. spicifera*) and *Fusarium* (*F. avenaceum,* and *F. graminearum*) collectively, comprised 13% of colonies. Of these, *B. sorokiniana* was the most abundant. The remaining colonies were species usually regarded as 'saprophytes' and included species of *Ulocladium, Botrytis, Stemphyllium, Nigrospora, Aspergillus,* and *Penicillium. B. sorokiniana* was recorded only in samples 2 and 4 but it was conspicuously more abundant in sample 2.

Evaluation of some seed-borne fungi on wheat

Seed discoloration

Analysis of the data Table (2) showed that there were significant differences between samples for both *Bipolaris* species and for *F. graminearum* (Table 2). Percentage scores for samples 1 and 2 (both cv. Yecora Roja) were very similar for all three fungi. The lowest scores for all three fungi were found on sample 4 (cv. Sammah) and these were significantly lower than on the other three samples for both *Bipolaris* species; for *F. graminearum* only cv. Logame (sample 3) differed from cv. Sammah.

Significant differences were detected between inoculation times for *A. alternata*, *B. sorokiniana* and *B. spicifera* but not for the two *Fusarium* species (Table 2). Differences between inoculation times for *A. alternata* and the two *Bipolaris* species are, however, mainly due to differences between I_3, the uninoculated control and I_1 and I_2, which, except for *B. sorokiniana*, did not differ from one another. Inoculation with *B. sorokiniana* at I_2 produced more discolored grains than at I_1 (Table 2).

More discolored grains were produced under wet than dry conditions (Table 2). Some discoloration (2- 3%) was found in the uninoculated control under wet conditions compared to a virtually complete absence of discoloration under dry conditions. Under dry conditions some discoloration was produced by *A. alternata* and the two *Bipolaris* species but under wet conditions much higher percentages were recorded especially with *Bipolaris* species accounting for the highly significant. For the *Fusarium* species very low scores were recorded even under wet conditions (Table 2). Interactions, samples x inoculation times and samples x conditions, were highly significant for the two *Bipolaris* species; for other species they were either not significant or only just significant (Table 2).

Seed shriveling

Significant differences between samples in the proportions of shriveled grains were found

Table 1. Mean number of fungal colonies per plate produced by four wheat samples

Fungi		Wheat Sample				Mean
		1	2	3	4	
Alternaria alternata		0.6	4.5	6.4	6.8	4.58
Bipolaris sorokiniana		0.0	2.4	0.0	0.1	0.63
B. spicifera		0.0	0.4	0.0	0.0	0.10
Fusarium avenaceum		0.0	0.3	0.0	0.0	0.08
F. graminearum		0.0	0.1	0.0	0.0	0.03
Others		0.3	1.4	2.2	0.3	1.05
Total fungal colonies after sterilization	Sodium hypochlorite	1.2	10.2	8.6	7.6	6.9
	Mercuric chloride	0.6	8.0	8.6	6.8	6.0

1: cv. Yecora Rojo (USA grown), 2: cv. Yecora Rojo (Saudi grown), 3: cv. Logame (Saudi grown) and 4: cv. Sammah (Saudi grown)

Table 2. Mean percentage of discolored grains in four samples of wheat inoculated with five fungi under wet and dry conditions.

Treatment		*Alternaria alternata*			*Bipolaris sorokiniana*			*B. spicifera*			*Fusarium avenaceum*			*F. graminearum*		
		W	D	Mean	W	D	Mean	W	D	Mean	W	D	Mean	W	D	Mean
Wheat	1	7.76	1.31	4.54	48.85	5.41	27.13	23.03	1.08	12.06	0.82	0.01	0.42	0.01	0.01	0.01
Sample	2	3.89	3.56	3.73	48.32	3.57	25.95	22.04	2.89	12.47	2.04	0.01	1.03	0.69	0.01	0.35
	3	6.64	2.42	4.53	58.35	18.02	38.19	22.69	5.90	14.30	1.64	1.44	1.54	2.60	0.49	1.55
	4	8.32	2.47	5.40	33.74	6.78	20.26	10.44	2.47	6.46	0.01	0.01	0.01	0.01	0.01	0.01
Inoculation	I_1	8.59	2.59	5.59	61.36	7.50	34.43	25.98	6.23	16.11	1.2	0.03	0.62	0.94	0.01	0.48
	I_2	8.61	4.74	6.68	78.23	16.76	47.50	28.60	3.03	15.82	1.8	0.5	1.15	1.01	0.02	0.52
	I_3	2.76	0.01	1.39	2.36	0.36	1.36	3.33	0.01	1.67	0.80	0.01	0.41	0.76	0.01	0.39
Analysis	S	0.29			31.67***			7.22***			2.73			3.70*		
of variance	I	6.48**			419.82***			58.95***			1.02			0.15		
	C	11.47**			550.59***			143.18***			5.23*			6.50*		
	S x I	0.58			6.87***			4.97**			3.73*			0.80		
	S x C	1.22			6.34***			4.77**			1.88			3.30		
	I x C	0.59			125.67***			24.19***			4.28*			0.14		
	S x I x C	0.76			3.15*			1.75			2.20			0.65		

-1: cv. Yecora Rojo (USA grown), 2: cv. Yecora Rojo (Saudi grown), 3: cv. Logame (Saudi grown) and 4: cv. Sammah (Saudi grown).
-S =sample, I_1= inoculation at anthesis, I_2= inoculation at anthesis + 12 days, I_3= control, C = conditions (wet/dry).
-* = low significant, ** = medium significant and *** = high significant at $p = 0.05$.

Table 3. Mean percentage of shriveled grains in four samples of wheat inoculated with five fungi under wet and dry conditions

Treatment		*Alternaria alternata*			*Bipolaris sorokiniana*			*B. specifera*			*Fusarium avenaceum*			*F. graminearum*		
		W	D	Mean	W	D	Mean	W	D	Mean	W	D	Mean	W	D	Mean
Wheat	1	20.24	1.76	11.0	42.76	0.68	21.72	21.35	0.97	11.16	18.33	0.01	9.17	20.49	1.80	11.15
Sample	2	18.76	2.72	10.74	38.74	0.01	19.38	19.02	2.96	10.79	15.85	1.37	8.61	21.42	4.58	13.00
	3	12.87	3.15	8.01	57.40	3.82	30.61	23.52	2.61	13.07	14.69	1.16	7.93	19.56	6.10	12.83
	4	3.30	1.93	2.62	14.16	0.01	7.09	5.09	0.48	2.79	5.79	1.16	3.48	13.20	4.03	8.62
Inoculation	I1	8.45	0.3	4.38	50.13	0.63	25.38	23.86	3.33	13.60	4.41	0.08	2.25	22.62	6.94	14.78
	I2	10.2	0.4	5.30	55.96	2.75	29.36	20.31	0.01	10.16	6.02	0.10	3.06	23.81	5.08	14.45
	I3	7.13	0.21	3.67	8.70	0.01	4.36	7.57	1.76	4.67	3.63	0.02	1.83	9.58	0.36	4.97
Analysis of variance	S		7.14***			19.25***			5.58**			3.53*	1.05			
	I		2.37			49.23***			7.25**			1.20			10.49***	
	C		126.40***			307.07***			75.93***			75.59***			72.58***	
	S x I		2.19			4.87**			0.96			1.88			1.22	
	S x C		14.18***			15.32***			4.52**			3.90*			1.51	
	I x C		0.83			45.30***			7.46**			1.23			2.70	
	S x I x C		0.94			4.00**			0.96			0.86			1.56	

-1: cv. Yecora Rojo (USA grown), 2: cv. Yecora Rojo (Saudi grown), 3: cv. Logame (Saudi grown) and 4: cv. Sammah (Saudi grown).
-S =sample, I1= inoculation at anthesis, I2= inoculation at anthesis + 12 days, I3= control, C = conditions (wet/dry).
-* = low significant, ** = medium significant and *** = high significant at p = 0.05.

Table 4. Mean weight (g) of 100 seeds of four samples of wheat inoculated with five fungi under wet and dry conditions

Treatment		*Alternaria alternata*			*Bipolaris sorokiniana*			*B. specifera*			*Fusarium avenaceum*			*F. graminearum*		
		W	D	Mean	W	D	Mean	W	D	Mean	W	D	Mean	W	D	Mean
Wheat	1	3.98	5.43	4.71	3.17	5.36	4.27	3.91	5.68	4.81	4.12	5.45	4.79	4.09	5.22	4.66
Sample	2	4.17	4.89	4.53	3.83	5.69	4.75	3.84	5.34	4.59	4.43	5.68	5.06	4.04	5.11	4.58
	3	5.10	6.51	5.80	3.31	6.62	4.96	4.92	7.48	6.21	5.41	7.69	6.55	4.99	7.38	6.19
	4	3.96	4.47	4.22	3.63	4.59	4.11	3.91	4.63	4.27	4.29	4.50	4.41	4.19	4.39	4.29
Inoculation	I1	3.36	5.86	4.61	3.23	5.01	4.12	3.38	5.08	4.23	4.45	5.81	5.13	4.17	4.75	4.46
	I2	3.12	5.76	4.44	3.31	4.85	4.08	3.13	5.09	4.11	4.23	5.51	4.87	4.08	4.54	4.31
	I3	4.29	5.65	4.97	4.02	5.00	4.51	3.87	5.67	4.77	5.87	7.15	6.51	4.67	5.11	4.89
Analysis	S		3.57*			2.83*			13.51***			1.01			2.36	
of variance	I		5.96**			5.41**			19.79***			1.09			4.76**	
	C		461.22***			110.22***			694.81***			38.17***			46.12***	
	S x I		1.29			1.11			2.57*			1.58			1.69	
	S x C		6.35**			2.58			11.42***			3.01			4.15*	
	I x C		6.39**			0.82			1.88			0.73			1.69	
	S x I x C		2.79*			1.87			3.54**			0.89			2.64*	

-1: cv. Yecora Rojo (USA grown), 2: cv. Yecora Rojo (Saudi grown), 3: cv. Logame (Saudi grown) and 4: cv. Sammah (Saudi grown).
-S =sample, I1= inoculation at anthesis, I2= inoculation at anthesis + 12 days, I3= control, C = conditions (wet/dry).
-* = low significant, ** = medium significant and *** = high significant at p = 0.05

Table 5. Mean percentage of ungerminated seeds in four samples of wheat inoculated with five fungi under wet and dry conditions

Treatment		*Alternaria alternata*			*Bipolaris sorokiniana*			*B. specifera*			*Fusarium avenaceum*			*F. graminearum*		
		W	D	Mean	W	D	Mean	W	D	Mean	W	D	Mean	W	D	Mean
Wheat	1	12.28	0.01	6.15	27.41	0.01	13.71	2.77	1.54	2.16	0.01	0.01	0.01	8.66	9.23	8.96
Sample	2	3.75	0.01	1.88	28.82	6.15	17.49	15.86	1.54	8.70	11.13	1.15	6.14	7.50	3.07	5.29
	3	16.97	3.07	10.02	47.51	35.45	41.48	18.52	13.61	16.07	20.16	9.42	14.79	22.44	11.80	17.12
	4	2.67	0.01	1.34	35.09	5.84	20.47	0.01	0.01	0.01	4.23	0.01	2.12	0.01	0.01	0.01
Inoculation	I1	12.44	1.15	6.80	49.08	20.13	34.61	15.46	7.90	12.68	9.50	5.91	7.71	10.29	10.66	10.48
	I2	4.38	0.01	2.20	46.61	13.14	29.88	9.18	1.15	5.17	13.18	2.31	7.75	13.69	7.42	10.56
	I3	9.93	1.15	5.54	8.44	2.31	5.38	3.23	3.46	3.35	3.97	0.01	1.99	4.98	0.01	2.50
Analysis	S		5.10*			67.50***			17.60***			13.13***			12.83***	
of variance	I		2.36			143.12***			8.69***			4.50*			7.09**	
	C		21.93***			196.78***			6.81*			10.14***			3.71	
	S x I		1.59			9.76***			5.03***			1.71			1.44	
	S x C		2.69			5.59**			2.81			1.67			1.90	
	I x C		1.34			26.97***			1.87			1.51			1.17	
	S x I x C		1.02			9.81***			0.85			0.87			3.87**	

-1: cv. Yecora Rojo (USA grown), 2: cv. Yecora Rojo (Saudi grown), 3: cv. Logame (Saudi grown) and 4: cv. Sammah (Saudi grown).
-S =sample, I1= inoculation at anthesis, I2= inoculation at anthesis + 12 days, I3= control, C = conditions (wet/dry).
-* = low significant, ** = medium significant and *** = high significant at p = 0.05.

Table 6. Mean seedling length (mm) of four samples of wheat inoculated with five fungi under wet and dry conditions

Treatment		*Alternaria alternata*			*Bipolaris sorokiniana*			*B. specifera*			*Fusarium avenaceum*			*F. graminearum*		
		W	D	Mean	W	D	Mean	W	D	Mean	W	D	Mean	W	D	Mean
Wheat	1	7.61	7.92	7.77	7.22	7.87	7.55	8.01	7.93	7.97	7.96	8.06	8.01	8.47	7.79	8.13
Sample	2	7.95	8.17	8.06	6.52	8.19	7.36	7.82	8.11	7.97	7.99	7.69	7.84	8.29	7.76	8.03
	3	10.88	10.48	10.68	6.47	8.87	7.67	10.39	10.43	10.41	10.13	10.01	10.07	10.70	10.32	10.51
	4	10.95	10.95	10.95	8.83	9.97	9.40	11.29	10.84	11.07	11.04	10.50	10.77	11.08	10.87	10.98
Inoculation	I1	8.12	8.02	8.07	6.19	8.65	7.42	8.22	8.08	8.15	9.49	9.36	9.43	9.36	9.10	9.23
	I2	8.81	8.53	8.67	6.31	8.09	7.20	9.27	8.23	8.75	8.91	8.66	8.79	9.57	9.25	9.41
	I3	9.14	9.10	9.12	9.28	9.45	9.37	9.46	9.20	9.33	9.04	9.18	9.31	9.67	9.57	9.62
Analysis	S		78.23***			28.48***			94.92***			107.68***			92.83***	
of variance	I		1.2			60.28***			0.59			7.62**			0.02	
	C		0.09			47.78***			0.09			3.57			20.38***	
	S x I		0.7			12.65***			1.22			2.95*			0.91	
	S x C		2.10			3.09*			0.96			1.46			1.03	
	I x C		3.13			10.19***			3.41*			0.14			6.34**	
	S x I x C		4.02			2.41*			0.97			1.03			1.44	

-1: cv. Yecora Rojo (USA grown), 2: cv. Yecora Rojo (Saudi grown), 3: cv. Logame (Saudi grown) and 4: cv. Sammah (Saudi grown).
-S =sample, I1= inoculation at anthesis, I2= inoculation at anthesis + 12 days, I3= control, C = conditions (wet/dry).
-* = low significant, ** = medium significant and *** = high significant at p = 0.05.

Table 7. Mean fungal colonies isolated from four samples of wheat inoculated with five fungi under wet and dry conditions

Treatment		*Alternaria alternata*			*Bipolaris sorokiniana*			*B. specifera*			*Fusarium avenaceum*			*F. graminearum*		
		W	D	Mean	W	D	Mean	W	D	Mean	W	D	Mean	W	D	Mean
Wheat	1	55.57	59.19	57.79	58.94	49.23	54.09	63.27	61.06	62.16	60.00	45.96	52.98	60.00	57.79	58.89
Sample	2	63.27	57.79	60.53	67.70	55.57	61.63	63.27	57.79	60.53	53.46	37.50	45.48	62.21	52.30	57.25
	3	55.57	56.73	56.15	63.37	44.70	54.04	63.27	55.57	59.42	57.79	32.02	44.90	63.27	56.73	59.99
	4	60.00	55.57	57.79	60.00	52.30	56.15	63.27	50.29	56.78	60.00	34.23	47.12	56.73	37.39	47.06
Inoculation	I1	85.02	82.27	83.79	87.55	70.45	78.99	90.00	83.36	86.68	83.44	51.35	67.39	87.55	74.34	80.95
	I2	83.36	88.34	85.85	85.09	76.79	80.95	90.00	77.88	83.94	90.00	60.94	75.47	90.00	78.82	84.41
	I3	7.43	1.66	4.55	14.87	4.11	9.49	9.80	7.29	8.55	0.001	0.001	0.001	4.11	0.001	2.06
Analysis	S	0.69	1.57	0.62	1.59	7.27***										
of variance	I		598.64***			271.58***			319.25***			267.19***			597.15***	
	C		0.22			20.91***			6.58*			63.45***			18.31***	
	S x I		0.74			2.4*			2.03			2.41*			1.32	
	S x C		1.02			0.79			0.67			1.49			2.68	
	I x C		1.87			0.97			1.01			15.98***			1.54	
	S x I x C		0.69			1.69			2.18			1.52			1.98	

-1: cv. Yecora Rojo (USA grown), 2: cv. Yecora Rojo (Saudi grown), 3: cv. Logame (Saudi grown) and 4: cv. Sammah (Saudi grown).
-S =sample, I1= inoculation at anthesis, I2= inoculation at anthesis + 12 days, I3= control, C = conditions (wet/dry).
-* = low significant, ** = medium significant and *** = high significant at p = 0.05.

Table 8. Mean glucosamine content (µg/g) in four samples of wheat inoculated with five fungi under wet and dry conditions

Treatment		*Alternaria alternata*			*Bipolaris sorokiniana*			*B. specifera*			*Fusarium avenaceum*			*F. graminearum*		
		W	D	Mean	W	D	Mean	W	D	Mean	W	D	Mean	W	D	Mean
Wheat	1	6.73	6.20	6.46	10.61	6.71	8.66	6.77	6.36	6.56	7.22	6.89	7.06	8.18	7.15	7.67
Sample	2	7.01	6.38	6.70	11.30	7.13	9.22	7.28	6.36	6.82	7.15	6.73	6.94	7.89	7.06	7.48
	3	7.19	6.82	7.01	23.04	7.20	15.12	8.14	6.68	7.41	9.09	6.68	7.89	10.65	7.43	9.04
	4	6.36	5.50	5.93	9.64	6.88	8.26	7.81	6.26	7.03	6.95	7.01	6.98	7.73	6.53	7.13
Inoculation	I1	7.63	6.80	7.22	15.64	8.24	11.94	7.97	6.87	7.42	9.00	7.98	8.49	9.28	7.55	8.42
	I2	7.66	6.98	7.32	18.98	7.58	13.28	8.54	7.57	8.06	9.31	7.18	8.25	10.73	8.14	9.44
	I3	5.17	4.89	5.03	6.31	5.12	5.72	5.78	4.81	5.40	4.50	5.33	4.92	5.82	5.44	5.63
Analysis	S		5.08**			120.83***			1.38			1.59			5.54**	
of variance	I		54.82***			251.75***			27.54***			41.52***			40.80***	
	C		5.56*			386.00***			18.71***			6.97*			23.65***	
	S x I		0.64			26.88***			0.93			0.95			0.86	
	S x C		.016			82.13***			1.11			3.55			2.95*	
	I x C		0.42			76.56***			0.054			8.59**			4.00*	
	S x I x C		0.7			21.09***			0.583			2.03			0.39	

-1: cv. Yecora Rojo (USA grown), 2: cv. Yecora Rojo (Saudi grown), 3: cv. Logame (Saudi grown) and 4: cv. Sammah (Saudi grown).
-S =sample, I1= inoculation at anthesis, I2= inoculation at anthesis + 12 days, I3= control, C = conditions (wet/dry).
-* = low significant, ** = medium significant and *** = high significant at p = 0.05.

Table 9. Mean ergosterol content (µg/g) in four samples of wheat inoculated with five fungi under wet and dry conditions

Treatment		*Alternaria alternata*			*Bipolaris sorokiniana*			*B. specifera*			*Fusarium avenaceum*			*F. graminearum*		
		W	D	Mean	W	D	Mean	W	D	Mean	W	D	Mean	W	D	Mean
Wheat	1	1.59	1.49	1.54	2.76	1.69	2.22	1.77	1.23	1.50	1.74	1.09	1.42	1.48	1.10	1.73
Sample	2	2.07	1.26	1.67	2.96	1.56	2.36	1.66	1.36	1.51	1.55	1.32	1.43	1.44	1.04	1.65
	3	2.06	1.71	1.89	3.57	1.85	2.71	2.17	1.65	1.91	2.35	1.54	1.95	1.95	1.28	2.15
	4	1.55	0.99	1.27	2.19	1.27	1.73	1.65	1.17	1.41	1.53	1.35	1.44	1.50	0.96	1.41
Inoculation	I1	2.02	1.60	1.81	3.35	1.80	2.58	2.02	1.44	1.73	1.97	1.25	1.61	2.34	1.56	1.95
	I2	2.13	1.41	1.77	3.64	1.81	2.72	2.06	1.51	1.79	2.23	1.54	1.88	2.55	1.62	2.08
	I3	1.31	1.08	1.19	1.62	1.16	1.39	1.36	1.10	1.23	1.18	1.18	1.18	1.13	1.20	1.16
Analysis	S		7.80**			36.86***			31.80***			15.77***			14.59***	
of variance	I		18.96***			163.12***			79.01***			39.65***			50.18***	
	C		35.80***			178.23***			24.91***			7.49*			51.81***	
	S x I		1.08			3.34*			2.03			1.79			1.47	
	S x C		3.85*			3.41			0.34			0.85			3.12	
	I x C		3.54			19.22***			1.19			1.93			16.78***	
	S x I x C		1.57			1.98			1.19			0.75			1.27	

-1: cv. Yecora Rojo (USA grown), 2: cv. Yecora Rojo (Saudi grown), 3: cv. Logame (Saudi grown) and 4: cv. Sammah (Saudi grown).
-S =sample, I1= inoculation at anthesis, I2= inoculation at anthesis + 12 days, I3= control, C = conditions (wet/dry).
-* = low significant, ** = medium significant and *** = high significant at p = 0.05.

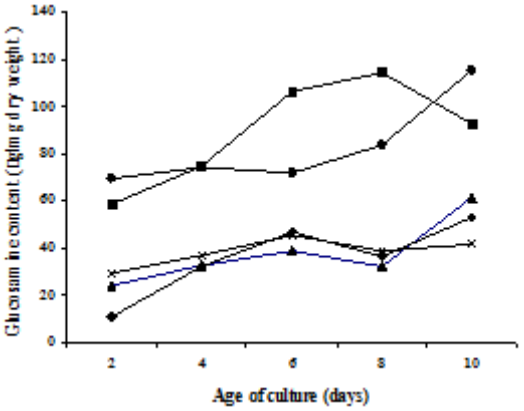
Table 10. Amount of mycelium (µg/seed) in inoculated seeds and uninoculated seeds (C) of four samples of wheat inoculated with five fungi.

Sample*		*Alternaria alternata*		*Bipolaris sorokiniana*		*B. specifera*		*Fusarium avenaceum*		*F. graminearum*	
		I**	C	I	C	I	C	I	C	I	C
Glucosamine	1	68.06	29.71	130.31	42.86	73.83	27.36	36.67	15.46	43.52	19.34
	2	67.68	31.95	168.30	46.52	71.56	33.17	38.69	12.40	43.38	15.21
	3	45.38	26.27	113.81	35.97	67.66	43.78	34.96	11.79	34.32	17.11
	4	66.59	37.41	264.6	50.05	79.16	43.96	44.69	15.31	51.09	21.19
Ergosterol	1	41.67	18.45	100.59	20.45	49.02	17.71	33.76	15.22	49.54	19.15
	2	53.49	15.76	113.44	33.37	42.67	22.88	34.27	15.31	44.16	18.99
	3	65.14	48.12	157.63	63.27	84.76	57.88	85.72	30.65	107.44	32.41
	4	25.06	12.30	55.86	19.33	35.28	18.28	21.99	14.15	32.25	9.56

* 1: cv. Yecora Rojo (USA grown), 2: cv. Yecora Rojo (Saudi grown), 3: cv. Logame (Saudi grown) and 4: cv. Sammah (Saudi grown).
** I = mean of inoculated seeds (I_1, I_2) and C= uninoculated seeds

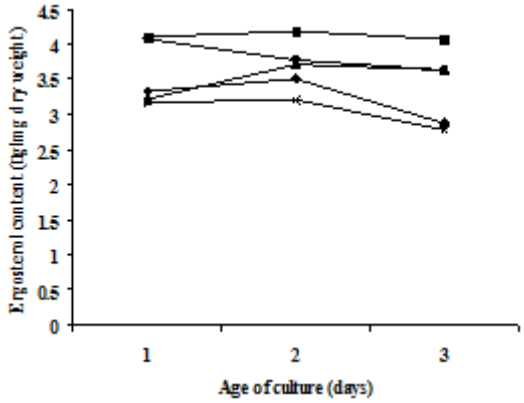
for all fungi except *F. graminearum* (Table 3). As noted for discoloration, differences between samples 1 and 2 (cv. Yecora Rojo) were small and not significant. Significantly lower scores for all fungi were found on sample 4 (cv. Sammah). Scores on sample 3 (cv. Logame) were, for all fungi except *B. sorokiniana* , similar to those on sample 1 and 2; on *B. sorokiniana* significantly higher scores were recorded (Table 3).

Significant differences between inoculation times were found for three species; *B. sorokiniana* , *B. spicifera*, and *F. graminearum*, (Appendix Table 3.2) but these differences were, in all instances, between the control (I_3) and I_1 and



(²%) *A. alternata*, (f&) *B. sorokiniana*, (×) *B. spicifera*, (Ï%) *F. graminearum* and (%) *F. avenaceum*

Fig. 1. Glucoseamine content of mycelium produced in pure culture



(²%) *A. alternata*, (f&) *B. sorokiniana*, (×) *B. spicifera*, (Ï%) *F. graminearum* and (%) *F. avenaceum*

Fig. 2. Ergosterol content of mycelium produced in pure culture

I_2, which did not differ from one another (Table 3).

As might be expected, more shriveled grains were produced under wet than dry conditions (Table 3) and even in the uninoculated control more shriveled grains were found under wet conditions. The interaction, samples x conditions (wet or dry), was significant for all fungi except *F. graminearum* and was particularly strong for *A. alternata* and *B. sorokiniana*.

Seed weight

All five fungi reduced seed weight under wet conditions in all samples, the largest reductions being produced by the two *Bipolaris* species (Table 4). As might be expected from the data for proportions of shriveled seed, reductions in seed weight differed considerable between samples. The mean reductions (%), (based on means of I_1 and I_2 under wet conditions; I_3 under dry conditions = 100) were: sample 1, 34; sample 2, 31; sample 3, 42 and sample 4, 17. Significant differences were found for three of the four samples (Table 4) but these arose almost entirely from the larger seed weight of the control (I_3) compared to I_1 and I_2 which only differed from one another in sample 3. There was an indication that the *Bipolaris* species were relatively less damaging under dry than wet conditions, the opposite being true for the *Fusarium* species. The interaction, inoculation times x conditions, only observed in sample 1 arose from a reversal of ranking of I_1 and I_2 in wet and dry conditions respectively (Table 4).

Seed germination

Differences in germination between samples were found in all fungal treatments (Table 5). Overall differences between samples 1 and 2 (cv. Yecora Rojo) were small and only just significant with *F. avenaceum*. The two Saudi Arabian cultivars were strikingly different: germination of cv. Logame (sample 3) was poor in all fungal treatments whereas germination of cv. Sammah (sample 4) was, with the exception of *B. sorokiniana*, very good (Table 5).

Germination was reduced by inoculation with all five fungi except *A. alternata* (Table 5). For the remainder differences between inoculation times were also significant, mainly because germination in the control (I_3) was significantly greater; I_1 and 1_2 differed little from one another with *Fusarium* species but with *Bipolaris* species there were fewer ungerminated seeds in I_2 than I_1 (Table 5).

Germination of seed produced under wet conditions was reduced even in the absence of fungal inoculation (Table 5) but was further reduced by inoculation with all fungi. Germination of seed from plants inoculated with *B. sorokiniana* was particularly poor and for this species the interactions, inoculations x conditions was highly significant (Table 5). Significant interactions between samples x inoculation times were found with *B. sorokiniana* and *B. spicifera* and between samples x conditions for *B. sorokiniana*.

Seedling growth

Seedling growth was assessed as plumule growth of those seeds which germinated. Samples 1 and 2 (cv. Yecora Rojo) did not differ in plumule length, the means for samples 1 and 2 being 7.89 and 7.85 respectively. Samples 3 and 4 had longer plumules, the overall means being 9.87 and 10.63 respectively. The slightly longer plumules of sample 4 (cv. Sammah) would appear to reflect its greater resistance to infection, particularly to *Bipolaris* species, than sample 3 (cv. Logame). Seed size was presumably not a significant factor since sample 4 had the smaller seed.

Only two fungal treatments (*B. sorokiniana* and *F. avenaceum*) showed significant effects of inoculation time (Table 6). For *B. sorokiniana* it is evident that the difference is due entirely to the higher value of the control (I_3). The results for *F. avenaceum* are curious in that whilst I_1 did not differ from I_3 plumule length in I_2 was significantly reduced.

Differences between seed produced under wet and dry conditions were small and only significant for samples inoculated with *B. sorokiniana* and *F. graminearum* (Table 6). Plumules produced from seed inoculated with *H. sativum* were shorter for the wet condition but the reverse was found with *F. graminearum*, where, it is interesting to note, a small increase occurred in all four samples (Table 6). The interaction inoculations x conditions was significant in three of the fungal treatments (*B. sorokiniana*, *B. spicifera* and *F. graminearum*).

Isolation of fungi from seeds

Data presented in (Table 7) showed highly significant differences between inoculation treatments for all five fungi used but, except for *F. avenaceum*, where I_2 was greater than I_1 (Table 7)

these derived from the differences between I_3 (uninoculated) and I_1 and I_2. More infected seeds were found in the wet than the dry environment although this difference was not statistically significant for *A. alternata*. No significant differences were found between samples except with *F. graminearum* where sample 4 (cv. Sammah) was less infected than the other three (Table 7). The significant interaction I x C for *F. avenaceum* (Table 7) derives from the similar levels of infection on I_3 under wet and dry conditions whereas in I_1 and I_2 there was more infection in wet than dry conditions.

Seeds from uninoculated control plants (I_3) showed a more varied flora than seeds from plants which had been artificially inoculated (I_1 and I_2). The data (Table 7) shows that cross contamination by species used for artificial inoculation was most common with *A. alternata* and the two *Bipolaris* species. It is also apparent that there was a trend for more infection in the dry than the wet environment, this being particularly evident with casual contaminants such as species of *Cladosporium, Penicillium* and *Aspergillus*.

Assay of chitin and ergosterol in mycelium of fungi grown in culture

Glucosamine was determined in mycelium of *A. alternata, B. sorokiniana, B. spicifera, F. avenaceum* and *F. graminearum*, harvested after 2, 4, 6, 8 and 10 days incubation (Fig. 1). Values for the two *Fusarium* species were greater than for the other three species, which differed little from one another. Amounts of glucosamine (µg mg^{-1} dry wt of mycelium) tended to increase during the incubation period (Fig. 1).

Ergosterol was determined in mycelium of the same fungal species, grown under the same conditions, but harvested after 2, 6 and 10 days (Fig. 2). Differences between species were much smaller, although again, the highest values were obtained with the two *Fusarium* species. Differences between sampling times were also much smaller (Fig. 2).

The main purpose of estimating glucosamine and ergosterol in mycelium produced in culture was to provide values which would enable mycelial contents of grain samples to be estimated. The extent to which mycelium in culture resembles mycelium in grain is unknown and in order to provide single values for both glucosamine and ergosterol for each fungus the decision was taken to use mean values based on all sampling dates.

Assay of glucosamine in seeds of wheat samples

Significant differences between samples were found with *A. alternata, B. sorokiniana* and *F. graminearum* (Table 8). Glucosamine content of samples 1 and 2 (cv. Yecora Rojo) were never significantly different. The lowest values were generally found on sample 4 (cv. Sammah) and the highest on sample 3 (cv. Logame) as shown in Table (8).

For all fungi, highly significant differences between inoculation times were recorded (Table 8). The uninoculated control (I_3) always contained significantly less glucosamine than either I_1 or I_2, which only differed significantly from one another with *B. sorokiniana* where I_2 was greater than I_1.

More glucosamine was found under wet than dry conditions (Table 8) the largest difference occurring with *B. sorokiniana*. The interaction, inoculations x conditions, was significant with *B. sorokiniana* and the two *Fusarium* species, although it was evidently much stronger with *B. sorokiniana*. The interaction, samples x inoculations, was highly significant for *H. sativum*; for other species it was either not significant or only just significant.

Assay of ergosterol in seeds of wheat samples

For each fungal species significant differences between samples were detected (Table 9). The ergosterol contents of samples 1 and 2 (cv. Yecora Rojo) were similar and did not differ significantly on any occasion. Sample 3 (cv. Logame) always had the greatest ergosterol content and sample 4 (cv. Sammah) the least except for *F. avenaceum*.

Significant differences between inoculation times were found for all fungi (Table 9). The lowest ergosterol concentrations were found in the uninoculated control (I_3). I_1 and I_2 did not differ significantly from one another except for *F. avenaceum* which had more ergosterol in I_2 than I_1 (Table 9).

For all fungi, grains produced under wet conditions contained significantly more ergosterol than grains produced under dry conditions (Table 9). The interaction, inoculations x conditions, which was significant for *B. sorokiniana* and *F. graminearum* (Table 9) are attributable to the small

amount of ergosterol found in uninoculated grains (I_3) under wet conditions. The interactions (samples x inoculations and samples x conditions) were either not significant or only just significant.

Estimation of mycelium in grain samples

Mean values of glucosamine and ergosterol in mycelium produced in pure culture were used to estimate the amount of mycelium in grain. Because amounts of both glucosamine and ergosterol in inoculation treatments I_1 and I_2 were only rarely significantly different from one another mean values of these two treatments were used. The data, as µg/seed are shown in Table (10).

DISCUSSION

A wide range of fungal species was isolated from the wheat samples but *A. alternata* was by far the most common accounting for 70% of all colonies. Species of *B. sorokiniana, B. spicifera, F. avenaceum,* and *F. graminearum* collectively, comprised 13% of colonies. These results were not unexpected. Many studies have shown that species of *Alternaria, Fusarium, Bipolaris* and *Cladosporium* are particularly common[17,4,18]. Failure to detect *Cladosporium* was unusual. *C. cladosporioides* is a major constituent of the air spora in the summer in temperate regions and is normally found amongst the fungi isolated from cereal grains.

All five fungi examined in this experiment are commonly isolated from discolored grain. Not surprisingly, therefore, inoculation, particularly under wet conditions, produced discoloration, although the pattern varied with species. Grains infected with *A. alternata* and *B. specifera* showed most discoloration toward the embryo and whereas in those infected with *B. sorokiniana* discoloration was much more extensive and shriveling was also much more severe with this species. There were clear differences between cultivars in terms of discoloration, shriveling and seed size. The two samples, 1 and 2, of cv. Yecora Rojo were always very similar and generally ranked between cv. Logame which was most susceptible, and cv. Sammah which was the most resistant. Fungal infection of grain is favored by moisture, particularly in the early weeks after anthesis[19].

Infection of grain by fungi may reduce its quality for milling. Discolored flour is obviously less acceptable for bread making, but additionally, many of the fungi involved (particularly species of *Alternaria* and *Fusarium*) are known to produce mycotoxins which are injurious and sometimes fatal to man and other animals. The quality of grain for consumption by man and animals can clearly be reduced by seed invading fungi but it should not be overlooked that the quality of the seed for use in agriculture may also be seriously affected. *Alternaria alternata*, a major cause of grain discoloration in cool, temperate areas has relatively small effects on germination and seedling vigor but the other species are potentially much more damaging. *B. sorokiniana* is evidently capable of radically reducing germination and vigor. and it is also capable of causing foot- rot in the crop.

Ergosterol is the principal sterol of fungal cell membranes and is either absent or a minor constituent in higher plants. It has been used as a chemical marker for measuring fungal biomass in airborne dust, building materials, soil, plant tissue, and grains[20]. Ergosterol also has been used to investigate fungal invasion in grains[21]. On the other hand, glucosamine, a growth indicator, has the advantage of being present only in fungal cells of some genera. This structure makes up the monomeric unit of the fungal cell wall[22] and it can be quantitatively estimated by depolymerization followed by the dosage of the released glucosamine. The level of glucosamine in fungi may vary according to the composition of the medium and the cultivation conditions[23], but it is always present in the microbial structure.

The use of estimates of glucosamine or ergosterol to measure the fungal content of plant tissues poses a number of problems. First, the assumption has to be made that the proportion of glucosamine or ergosterol in mycelium grown in pure culture is the same, or approximates closely to, that of mycelium developing in plant tissue. There seems to be no way of checking this assumption and the problem is further complicated by the fact that the amounts of both glucosamine and ergosterol per g of mycelium vary with age of culture. Selecting a value to estimate the fungal content of plant tissue is, therefore, arbitrary and a further potential source of error. In these studies mean values of glucosamine and ergosterol derived from mycelium in cultures of different age were used.

A second important assumption is that the glucosamine and ergosterol being measured are strictly of fungal origin. The assumption appears to be valid for ergosterol and it is interesting to note that in a study by Marin *et al*.[24], 16 species of food spoilage fungi were examined and they concluded that ergosterol content and colony diameters were better correlated to fungal biomass than fungal counts were. By contrast, assessments based on glucosamine may be complicated by the presence of plant glycoproteins containing glucosamine and N-methyl-D-glucosamine[25]. The amounts of such proteins in seeds vary considerably. In these experiments estimates of glucosamine in uninoculated control samples were in the range 20-40 ug/g. Further, the control samples, although not showing significant discoloration or shriveling, were by no means completely free of infection as evidenced by the frequency with which fungi were isolated in plating tests. Some of the glucosamine in these samples must have been contributed by fungal mycelium so that any contribution from host tissue would have been very small. Nevertheless, the possible effect of seed glycoprotein cannot be overlooked. Despite these problems, it is encouraging that estimates of mycelium in seed obtained by both ergosterol and glucosamine assay were well correlated particularly when estimates were expressed as ug mycelium/seed.

For some fungal species (*A. alternata, B. sorokiniana* and *B. spicifera*) glucosamine estimates provided higher values than ergosterol whilst for the others (*Fusarium* species) there was close agreement. If the difference between the two methods found with *A. alternata* and the two *Bipolaris* species were due to seed glycoproteins a similar difference would also be expected with the *Fusarium* species. It seems reasonable to conclude, therefore, that seed glycoproteins were not, in this experiment, a serious confounding factor. The reason why *Fusarium* species differed from the others is not clear but the most plausible explanation is that the glucosamine or ergosterol content of mycelium in grain differed from that in culture for *A. alternata* and the two *Bipolaris* species but not for the *Fusarium* species.

It is now necessary to turn to a consideration of the relationship between visual effects of fungal infection (discoloration and shriveling) and the fungal content of grain. Some relationship would be expected. In these studies, for example, grain produced under wet conditions showed greater discoloration and shriveling and greater amounts of mycelium as indicated by greater amounts of ergosterol and glucosamine. Further, although seeds infected with *A. alternata* were badly discolored whilst those infected with *F. avenaceum* were relatively undiscolored, more mycelium was present in grains infected with *F. avenaceum*. With *F. graminearum* where the estimated amount of mycelium was slightly higher than with *F. avenaceum* there was no indication of a correlation with discoloration. It is evident that visual characteristics of seed provide an imperfect guide to the extent that seed is colonized by fungi. This has important implications.

Many seed-invading fungi (notably species of *Fusarium* and *Alternaria*), are known to produce a variety of mycotoxins capable of inducing muscle spasms, vomiting and even death in man and certain non-ruminant animals[26,27,28].It would seem, therefore, that screening of grain samples for fungal infection may become increasingly important, particularly in wet seasons. Chemical analysis for either glucosamine or ergosterol, if developed for routine use, could well be helpful in identifying potentially dangerous samples on which additional analyses for specific mycotoxins could be carried out.

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