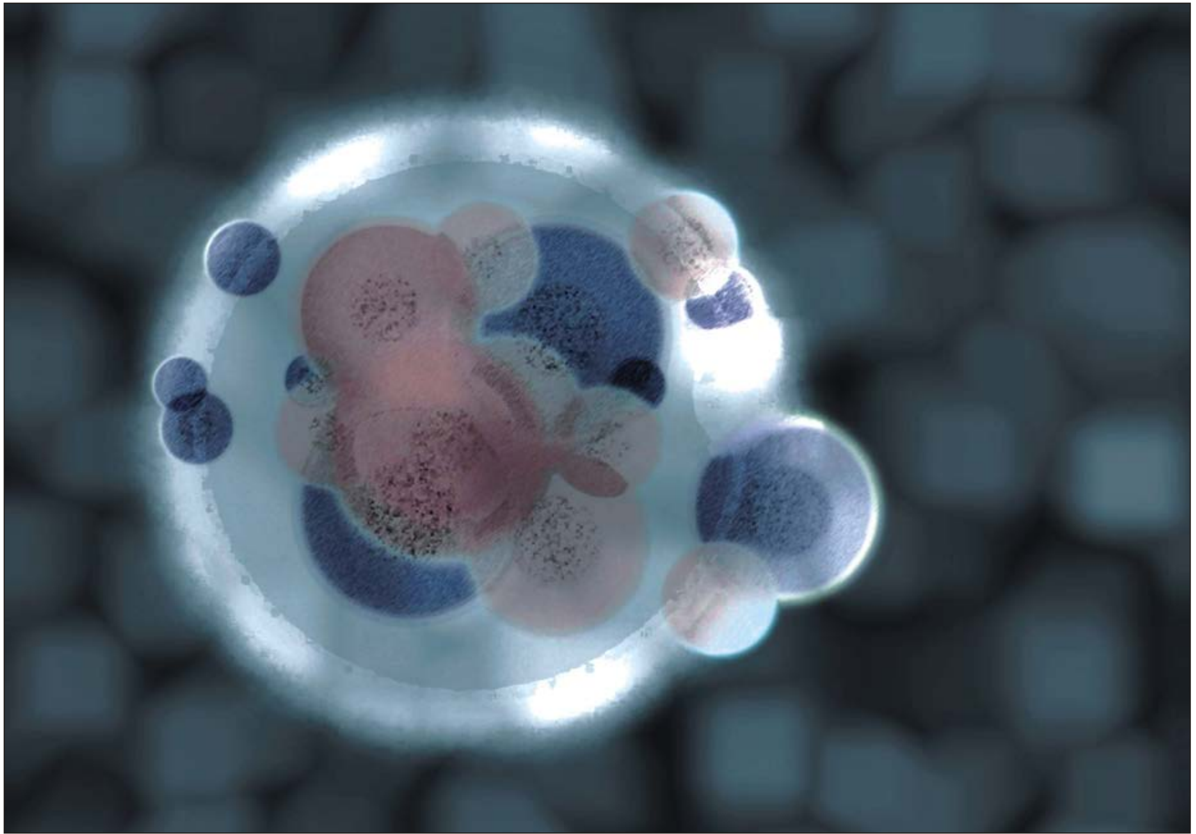

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Investigation of Fungal Species Diversity of Maize Kernels

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Abstract: This study was carried out for twenty retail and bulk maize samples parallel for each group, surface disinfected and non-disinfected maize kernels, in Balikesir, Turkey. The aim of the study has provided information to compare species diversity between non-disinfected and disinfected maize mycoflora. *Rhizopus* (49%) and *Aspergillus* (19%) were the most frequent genera isolated in non-disinfected maize kernels. Three species of *Rhizopus* spp. were commonly isolated; *R. oligosporus* Saito (19.0%), *R. oryzae* Went and Prinsen Geerlings (8.1%) and *R. stolonifer* (Ehrenb.) Lind. (22.0%). *Aspergillus* was the second most frequent genus isolated from non-disinfected maize kernels. Predominant species isolated were *Aspergillus tubingensis* (Schöiber) Mosseray (4.6%) and *A. niger* Van Tieghem (23%). In the disinfected group, *Aspergillus* spp. (25%), *Fusarium* spp. (21%), *Rhizopus* spp. (21%) and *Penicillium* spp. (13%) were commonly isolated. *Aspergillus tubingensis* (5.0%), *A. foetidus* var. *acidus* Naka, Simo and Wat (5.0%), *Fusarium proliferatum* (Matsushima) Nirenberg (17.1%), *Rhizopus oligosporus* (57%) and *Penicillium oxalicum* Currie and Thom (7.6%) were most frequently species isolated. Decrease of the *Rhizopus* genus by chlorine disinfection caused significantly increase of the *Fusarium* (21%), *Trichoderma* (8%) and *Aspergillus* (25%) rates. *Fusarium proliferatum* was also found dominant and potential mycotoxigenic "storage fungi" in the samples of corn maize.

Key words: *Aspergillus*, contamination, *Fusarium*, maize, *Penicillium*

INTRODUCTION

Maize (*Zea mays* L.) is the most important crop for animal feed and for human use in Turkey. Some factors such as, moisture, temperature, rainy days in a year during field stage and post harvest stage effect the crop and may cause fungal contaminations and loss of quality in the yield. Fungi are frequent contaminants of maize and their occurrence in feed brings about a decrease of the storage life of the product and represents a danger of occurrence of mycotoxinsand undergoes a change during the production and the storage of a foodstuff (Pitt and Hocking, 1997, Vytrasova *et al*., 2002). Grain molding is one of the most important factors of the deterioration of the quality of stored maize. The infection of grains by several mycotoxin producing capability fungi can cause serious hazards for human and animal health (Miller, 1995; Petzinger and Weidenbach, 2002). Most of food-spoiled fungi belonging to Deuteromyetes have the capability of producing toxic metabolites (Mego and Hayes, 1973; Blumenthal, 2004).

Balikesir, on the west of Turkey, 220 m elevation, has a typical eumediterranean climate; arid in the summer, inconsiderable rainy and cold in the winter. Average annular precipitation was 559.3 (2002), 476.8 (2003) and 506.2 (2003) mm/year average temperature was 14.4°C (2002), 13.9°C (2003) and 14.7°C (2004). Maximum temperature values were 43.2°C in August (2002), 41.8°C in July (2003) and 41.8°C in August (2004), respectively (Ministry of Environment and Forest, 2005) (Table 1).

Table 1: Average annual meteorological values of Balikesir

Average annual meteorological values of Balikesir	Years	Temperature (C)	Precipitation (mm/year)	Humidity (%)
Mean of the years	2002	14.5	559.3	72.42
	2003	13.9	476.8	73.2
	2004	14.7	506.2	70.51
Extreme (max/min) values	2002	43.2 August -10.2 January	79.1 November	82.1 November
	2003	41.8 July -7.4 February	78.2 February	87.6 November
	2004	41.2 August -18.8 February	172.5 November	84.6 November

There is no more available information on incidence of toxigenic fungal species associated with Turkey's grains including maize, which are produced and consumed widely. The aim of this study is to bring to light the incidence and the significance of maize mycoflora under storage conditions in Balikesir, Turkey.

Our knowledge about fungal flora of maize commodity was to determine potential mycotoxigenic species and to evaluate the results for the future. This paper presents our comparison analyses of fungal flora between non-disinfected surface and disinfected surface of maize.

MATERIALS AND METHODS

A total of twenty samples were collected and all of them were not less than 1-2 kg-size. Ten collected samples were taken from representing the maize lots between five and twenty tons or from big sacks in the grain depots. Four samples were taken at random from outlets and six samples were taken from the bazaars in Balikesir, Turkey. This study was carried out at following steps; a) Samples were collected from grain depots, outlets and bazaars between 2002 and 2004. b) a_w and water content values of each sample were determined just after being taken. c) Isolations were performed for each sample of disinfected maize mycoflora and non-disinfected maize mycoflora. d) Fungi associated with corn were identified. e) Distribution of fungal species and their potential mycotoxin producing capability was evaluated. f) Correlation between a_w and water content was evaluated.

Since adhering spores on the surface of maize kernels can cause infectious and invasion when they find any opportunities, our aim was to compare non-disinfected and disinfected maize mycoflora in the same samples. This will allow us to compare species diversity in both mycoflora between surface contamination and internal invasion. We preferred a common used simple technique. The main risk of this technique was that other fungi might have been covered on the surface of the plate. Commercial chlorine bleach to remove adhering spores was used and determined internal invasion in disinfected group.

Water content and a_w of samples were determined after having been taken to the laboratory. All the samples were held -20°C for 72 h to remove mites and insects. Normal flora was determined from non-disinfected maize samples and so was disinfected flora determined after disinfections of samples by 1% commercial chlorine bleach for 2 min.

To prepare surface disinfected grains, grains of 50 g was held in 300 mL beaker and added 1% commercial chlorine bleach for 2 min and hurled in both way delicately then rinsed with sterile distilled water three times to remove chlorine gas then plated directly (approximately 5 g per plate) onto 10 plates surface. To prepare non-disinfected grains, the samples in this group were not treated with chlorine bleach.

Determination of a_w and water content, actual a_w values of all the samples were measured at 25°C using a_w sprint Thermoconstanter TH-500, Novasina, Zurich, Switzerland). In order to monitor the changes in aw over storage time, the measurements were repeated three times. The moisture content of the maize grains was determined immediately after sampling.

Antibiotic solutions were prepared and stock solutions were kept in refrigerator in dark and renewed every week.

Two different media, Dichloran Rose Bengal Chloramphenicol Agar (DRBC), (Difco 0587) (King *et al*., 1979; Jarvis, 1973) and Dichloran 18% Glycerol Agar (DG18) (Hocking and Pitt, 1980) for xerophylic fungi were used for isolation and enumeration of fungi from maize kernels. Czapex dox agar (CZ) (Oxoid CM97), Malt Extracts Agar (MEA) (Oxoid CM59) and Potato Dextrose agar (PDA) (Merck 110130) were used for identification. DRBC Agar was prepared by adding Chloramphenicol, (50 mg L^{-1} before autoclaving) and chlortetracycline (50 mg L^{-1} filtered to sterilise and added just before pouring to the plates) (Pitt and Hocking, 1997; Deak *et al*., 2001). Poured plates were dried overnight before inoculating.

For isolations and identifications of fungi, sub samples were prepared to form representative sample for each of them. In disinfected group, 50 g of sub sample was weighted, kernels were put into sterile beaker and disinfected by adding 1% commercial chlorine bleach for 2 min by hurling in both sides delicately then rinsed with sterile distilled water for a few times to remove chlorine after then dried between sterilized boldface towel paper. Kernels were plated directly (approximately 5 g per plate) on 10 general and selective media onto DRBC Agar plates and DG18 Agar plate's surface. For non-disinfected kernels, the procedure was the same apart from disinfections stage. All the plates were incubated at 27°C for 4-6 days for DRBC and DG18 agar, respectively. Fungal colonies were cultured on Czapex Dox agar; Malt extracts agar and Potato Dextrose agar for identification.

Fungal colonies were selected for identification according to the methods proposed for each fungus. Identification of *Aspergillus* was done according to Raper and Fennell (1965), Samson *et al*. (1981), Klich (2002). *Penicillium* genus identification was carried out according to Raper and Thom (1949), Samson and Pitt (2000) and Samson and Hoekstra (2004). For Deuteromycetes identifications (Domsch *et al*., 1980; Fassatiova, 1986; Hasenekeoğlu, 1991) were used. Except these identification keys, (Samson and Pitt, 1990; Von Arx, 1974; Pitt, 1979)were also used.

Identification of Fusarium proliferatum, sequencing of regions of taxonomical interests (ITS1, ITS2 and translation elongation factor α1), were evaluated by MUCL Culture Collection in BCCM.

Relative Density (RD) was calculated for each species or genus in each group as the number of isolates or genus/Total number of fungi isolated×100 (Pacin *et al*., 2002).

RESULTS

a_w and water contents of maize samples were shown in Table 2. While sample 6 had the highest a_w and the highest water content value among the samples, sample 2 and 3 were determined to have the least a_w and the least species diversity within the samples.

In order to reveal species diversity between non-disinfected and disinfected groups, we compare the species dominancy and conditions of water content and a_w of the samples.

Table 2: Water activity and water content of samples

Sample No	Water Content	Water activity	Sample No.	Wate Content	Water activity
1	10	0.382	11	13.4	0.563
2	8.2	0.303	12	12	0.642
3	9.8	0.333	13	11.2	0.457
4	10.8	0.373	14	13	0.511
5	14.6	0.59	15	12	0.469
6	17.2	0.723	16	13	0.528
7	13.8	0.627	17	12	0.502
8	12.8	0.523	18	15	0.603
9	16.6	0.387	19	11	0.475
10	12	0.465	20	8.2	0.491

Table 3: Distribution of mould genera in 20 maize samples

Moulds	%RD from non-disinfected samples	%RD from disinfected samples
Rhizopus	49	21
Aspergillus	19	25
Fusarium	7	21
Penicillium	16	13
Trichoderma	1	8
Cladosporium	3	2
Mucor	1	3
Other	3	7

Table 4: Frequency (%) of occurrence common species in both, non-disinfected and disinfected, group

Common species	Relative density (%)	
	Non-disinfected group	Disinfectected group
Acremonium stricticum	1.1	1.9
Acremonium zonatum	1.1	0.6
Aspergillus awamori	1.1	2.5
Aspergillus flavus	2.3	4.5
Aspergillus flavus var. *columnaris*	1.1	1.9
Aspergillus foetidus var. *acidus*	2.3	5.0
Aspergillus foetidus var. *pallidus*	3.5	3.2
Aspergillus tubingeusis	4.6	5.0
Aspergillus wentii	1.1	0.6
Fusarium proliferatum	7.0	17.1
Penicillium expansum	1.1	0.6
Penicillium oxalicum	7.0	7.6
Penicillium turbatum	4.6	2.5
Rhizopus oligosporus	19.0	5.7
Rhizopus oryzae	8.1	2.5
Rhizopus stolonifer	22.0	5.0
Trichoderma harzianum	1.1	3.2
Total spp.	88.3	69.4

Totally 243 isolates were obtained from 20 samples after isolation. Twenty three species were identified from 86 isolates in non-disinfected group. Thirty eight species were identified from 157 isolates in disinfected group (Table 3).

There seventeen species were in common in both group in 243 isolates while common species rate in non-disinfected group was 88%, it was 69% in disinfected group (Table 4).

While species diversity except common species was approximately 12% in non-disinfected group with 6 species (26%) in total 86 isolates, it was approx. 31% in disinfected group with 21 species (55%) in total 157 isolates.

Based on isolation frequency as well as relative density, the members of the *Rhizopus* were the most prevalent species (49%) and followed by *Aspergillus* (19%) and *Penicillium* (16%) in non-disinfected maize kernels during three years (Table 3). Members of the *Fusarium* ve *Trichoderma* were seen in low rate (7 and 1%), respectively.

In the non-disinfected group, *Rhizopus* was the most frequent genus isolated. The percentage of contamination of the genus in total was 49% in this group. In the genus *Rhizopus*, three species of *Rhizopus* were identified. *R. oligosporus* (19.0%), *R. oryzae* (8.1%) and *R. stolonifer* (22.0%). *Aspergillus* was the second most frequent genus isolated in non-disinfected group. The percentage of contamination was 19% (Table 5).

Seven species of Aspergillus were identified. The predominant species isolated were *Aspergillus tubingensis* (4.6%), *A. niger* (2.3%) and *A. foetidus* var. *pallidus* (3.5%). *Penicillium* genus (16%) was in third row in the frequency of isolation. Four species of Penicillium were identified. *P. oxalicum*, (7.0%), *P. turbatum* (4.6%) *P. crustosum* (3.4%)and *Penicillium expansum* (1.1%).

In the disinfected group, *Aspergillus* was the most frequent genus isolated. The percentage of contamination was of the genus in total was 25% in disinfected group. In the genus *Aspergillus*, twelve species of *Aspergillus* were identified. The predominant species isolated were *Aspergillus tubingensis* (5.0%), *A. foetidus* var. *acidus* (5.0%), *Aspergillus flavus*, (4.5%) and *A. niger* (2.3%).

Fusarium and *Rhizopus* were the second most frequent genera isolated in non-disinfected group. The percentage of contamination of *Fusarium* was 21%. Three species of *Fusarium* were isolated. *Fusarium proliferatum* (17.1%) was dominant in this genus and followed by *F. oxyporum* (3.18) and *F. semitecticum* (0.63%). With the percentage of contamination of *Rhizopus* (21%) was the same frequency in this group. *Rhizopus oligosporus* (5.7%), *Rhizopus stolonifer* (5.0%) and *Rhizopus oryzae* (2.5%). *Penicillium* genus ranked

Table 5: RD (%) of species diversity except common species between disinfected and non-disinfected groups

Non-disinfected group	RD (%)	Disinfected group	RD (%)
Aerobasidium pullulans var. *melanogenum*	1.1	*Aspergillus flavo-furcatis*	0.6
Aspergillus niger	2.3	*Aspergillus foetidus*	0.6
Cladosporium herbarum	1.1	*Aspergillus parasiticus*	0.6
Cladosporium sphaereospermium	2.3	*Aspergillus terreus var. americanus*	0.6
Mucor rasemosus	1.1	*ladospor cladosporoides*	1.9
Penicillium crustosum	3.4	*Fusarium oxyporum*	3.1
		Fusarium semitectum	0.6
		Mucor circinelloides	1.2
		Mucor hiemalis	0.6
		Mukor circinelloides f. Jeussenii	0.6
		Penicillium brevicompactum	1.2
		Penicillium italicum	0.6
		Penicillium corylophilum	0.6
		Rhizopus oryzae	2.5
		Rhizopus stolonifer	5.0
		Trichoderma harzianum	3.1
		Trichoderma viride	1.2
		Other	4.0
Total spp. (approximately)	12		31

Table 6: Species, which mycotoxin producing capability, isolated from non-disinfected and disinfected maize samples

Mycotoxin producer species	Non-disinfected samples	RD (%)	Disinfected samples	RD (%)	Mycotoxins
Aspergillus flavus	2	2.30	7	4.45	Aflatoxin B1, B2
Aspergillus awamori	1	1.16	4	2.54	Ochratoxin-A
Aspergillus foetidus			1	0.63	Ochratoxin-A
Aspergillus foetidus var. *pallidus*	3	3.48	5	3.18	Ochratoxin-A
Aspergillus niger	2	2.30			Ochratoxin-A
Aspergillus wentii	1	1.16	1	0.63	Ochratoxin-A
Fusarium proliferatum	6	6.97	27	17.19	Fumanisin B1, B2
Fusarium oxyporum			5	3.18	Fumanisin B1, B3
Total	15	17.37	50	32	

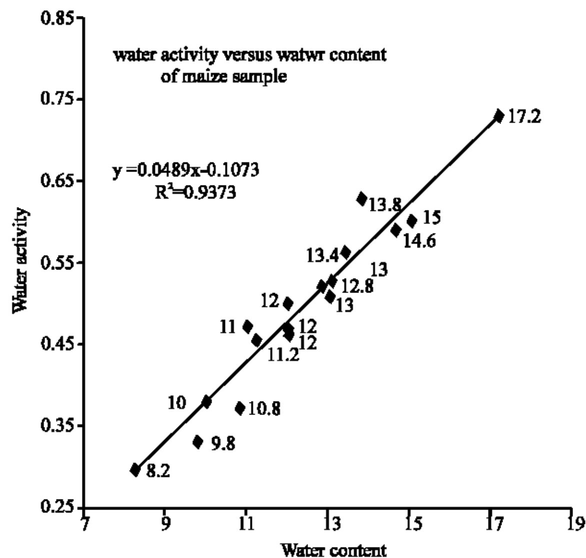

Fig. 1: Water activity versus water content of maize samples

fourth in the frequency of isolation. The percentage of contamination was 13%. *Penicillium oxalicum* (7.6%), *Penicillium turbatum* (2.5%) and *Penicillium expansum* (0.6%). Although, these *Penicillium* species are not mycotoxin producer, they might have an important role as biodeteriogen in stored maize kernels.

When water contents and a_w were taken into account, the results indicated that there was strong correlation (R^2 = 0.9373) between water contents and a_w (Fig. 1). In order to reach correct correlation between a_w and water content, three deviated data (9, 12 and 20) were ignored during linear regression.

Whereas, *Aspergillus* flavus, Aspergillus *awamori*, *Aspergillus foetidus var. pallidus*, *Aspergillus foetidus*, *Aspergillus niger*, *Aspergillus wentii*, *Fusarium proliferatum* were found as mycotoxin producing species in both group, *F. oxyporum* and *Aspergillus foetidus* were found only in disinfected group. *F. proliferatum* was one of the most frequently isolated species in disinfected group associated with maize.

DISCUSSION

In summary, the present study has provided information to compare species diversities in both mycoflora between surface contamination and internal

invasion. It is drawn a conclusion from this that, plate surface had been covering by fast developing *Rhizopus* spp. during a weekly-incubation period. It causes the other species to be invaded by *Rhizopus* spp. It is the reason that we preferred to isolate the species after 4-5 days incubation period.

When disinfected mycoflora of maize grains compare to non-disinfected flora, this has shown that Rhizopus spp. significantly inhibited growth of *Fusarium* (7%), *Trichoderma* (1%) and *Aspergillus* (19%) rates. Decrease of the *Rhizopus* genus by chlorine disinfection has caused significantly the increase of the *Fusarium* (21%), *Trichoderma* (8%) and *Aspergillus* (25%) rates (Table 3). The present work has been focused on disinfected and non-disinfected conditions.

Fusarium development occurred non-disinfected natural mycoflora of maize was inhibited and invaded by other fungi. It was observed that *Fusarium* species developed better in disinfected maize groups as some other researches supported this data (Marin *et al*., 1998 a, b and c).

While the percentage of common species in non-disinfected group was 88%, this rate was found as 69% in disinfected group. Diversity between two groups was found as 6 species for non-disinfected group and 21 species in disinfected group. Although, for only this study, statistical result shows that there are not significant ($p>0.05$) relationship between two groups in species diversity, however, RD of species and species diversity in both groups are clearly different from each other. We think that, to study more samples might cause to increase this species diversity and RD to the advantage of disinfected samples. We believe that this study will make a good starting step for other researchers as well, on this matter.

Potential mycotoxigenic fungi associated with maize kernels that might be of great importance such as *Aspergillus flavus* (Jiujiang *et al*., 2004), *Aspergillus awamori* (Varga *et al*., 1996), *Aspergillus foetidus* (Abarca *et al*., 1997), Aspergillus *foetidus var. pallidus* (Askun, 2002), *Aspergillus niger* (Abarca *et al*., 1994), *Aspergillus wentii* (Varga *et al*., 1996), *Fusarium proliferatum* (Dantzer *et al*. 1996) and *F. oxyporum* (Kpodo *et al*., 2000) (Table 6).

That *F. proliferatum* is natural contaminant of maize is known (Placinto, 1999). *F. proliferatum* was one of the most frequently isolated species in disinfected group associated with maize. *Fusarium* isolates were also found as dominant fungi by Orsi *et al*. (2000) in their study on maize. Pitt (2000) showed that Fumonisins are formed by only *F. proliferatum* but only in maize. In this study, we isolated *F. proliferatum* in storage stage fungi.

Since moisture control is an important factor for both developing fungi and producing mycotoxins, keeping commodity under low a_w is of great importance in tropic and subtropical regions. The greatest efforts should be shown to reduce a_w and water content in storage grain in order to prevent fungal development in food and feed.

Although earlier studies referred that *Liseola* section was the unique Fumonisins producing section in the genus *Fusarium* by *F. monilioforme, F. proliferatum, F. subglutinans* and *F. anthopilum* (Thiel *et al*., 1991), but, other studies have also shown that not only section *Liseola* but also section *Elegans* are capable of fumonisin (monilioformin) production by *F. oxyporum* (Placinta *et al*., 1999; Kpodo *et al*., 2000). Moreover, some *Fusarium* species such as *F. dlamini*, *F. nygamai* and *F. napiforme*, have been added as fumonisins producing species (Nelson *et al*., 1994).

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