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Investigation of stored wheat mycoflora, reporting the *Fusarium* cf. *langsethiae* in three provinces of Iran during 2007

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Abstract - Wheat is the most important cereal produced in Iran. A mycological survey was carried out for the first time, on the stored wheat samples in Tehran, East Azarbayejan and Mazandaran provinces in 2007. Exogenous and endogenous fungi, were isolated by the method of flotation with Malachite green agar (MGA 0.25) and Freeze blotter techniques respectively. In this study, 46 species belonging to 23 different genera were isolated. *Cladosporium* spp. (57.1-89.2%) and *Alternaria* spp. (82.4-100%) species were the predominant fungal species identified as endogenous mycoflora. The predominant exogenous fungi were *Penicillium* spp. (78.4-92.8%) and *Aspergillus* spp. (71.4-85.7%) species. *Fusarium proliferatum* was the most prevalent species of *Fusarium* isolates. *Aspergillus niger* (39.4%) and *Aspergillus flavus* (36.7%) were the predominant *Aspergillus* species identified as exogenous mycoflora. *Aspergillus flavus* (26.6%) was the predominant *Aspergillus* species identified as endogenous mycoflora. Flotation method with MGA 0.25 recommended for isolating of hyaline fungi from wheat cereals. In this study one isolate from *Fusarium* species was isolated on the basis of morphology and ribosomal internal transcribed spacer classified as *Fusarium langsethiae* but on the basis of partial translation elongation factor-1alpha gene grouped with *Fusarium sporotrichioides*. To our knowledge, this is the first report about *F.* cf. *langsethiae* in Iran and Asia.

Key words: wheat; *Fusarium* spp.; *Fusarium langsethiae*; *Aspergillus* spp.; flotation method.

INTRODUCTION

Wheat is an economic and important crop that provides approximately 20% of food calorie in the world (Wiese, 1987). It is one of the main staple foods in developing countries and the aspects of production and consumption is the most important grain in Iran (FAO, 2001). Bread wheat (*Triticum aestivum* L.) is the main cereal used for human consumption in Iran (Farshadfar *et al*., 2008). Information about fungi associated with these grains is important in assessing the risk of mycotoxin contamination. A number of fungal species, belonging mainly to the genera *Fusarium*, *Aspergillus* and *Penicillium*, have been reported to produce mycotoxins that cause serious illness and immunorepression in humans and animals (Marasas, 1995). This moulds enter the food chain in the field and during storage after harvest (Petzinger and Weindenbach, 2002).

There is more information concerning wheat mycoflora on the field and fresh stored wheat both on the international and national levels (Zamani-Zadeh and Khorsandi, 1995; Zare and Ershad, 1997; Walker *et al*., 2001; Bottalico and Perrone, 2002; Torp and Nirenberg, 2004; Darvish Nia *et al*., 2006; Davari *et al*., 2006; González *et al*., 2008). There are few reports concerning wheat mycoflora in store compared with field, and the most common fungi so far reported that contaminating wheat and flour in Australia are *Aspergillus* and *Penicillium* species (Berghofer *et al*., 2003). But the most common dematiaceous fungi so far reported in Brazil and Germany includes *Alternaria* spp. and *Cladosporium* spp. (Furlung *et al*., 1995; Weidenboner *et al*., 1997).

The relevant study in Iran has been performed in limited range. The report from the first relevant examination on stored wheat performed in location in central province of Iran is indicating that the most abundant species are *Cladosporium* and *Alternaria* (Saberi-Riseh *et al*., 2004). In other studies, *Aspergillus* has been presented as the most dominant contaminating species concerning stored wheat (Hedayati and Mohammad pour, 2005).

There are methods and media for isolation of fungi such as "Blotter" and "Agar plate" methods. The selective media for cultivation of fungi include Malachite green agar (MGA 0.25) and Nash and Snyder medium contain pentachloronitrobenzene (PCNB) (Castella *et al*., 1997a; Bragulat *et al*., 2004). The flo-

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tation method has been used for isolation of pathogenic fungi from soil (Smith and Furcolow, 1964; Vanittanakom *et al*., 1995). Our results based on the flotation method performed on soil samples from Isfahan province indicate that the predominant fungi include *Aspergillus*, *Penicillium* and *Fusarium* species (Kachuei *et al*., unpublished data). The selective medium Malachite green agar has been presented for the isolation of *Fusarium* species, specially for the isolation of *Fusarium verticilloides*. This medium relative to media contain PCNB, which is potentially carcinogen, has lesser risk (Castella *et al*.,1997a, 1997b; Bragulat *et al*., 2004). The flotation method accompanied with Malachite green agar so far has not been used for isolation of fungi from grains.

Fusarium langsethiae described as a new toxigenic *Fusarium* species (Torp and Nirenberg, 2004), which has been isolated from oats, wheat and barly in northern, central and east Europe (Norway, Austria, Germany, The Netherlands, Czech Republic, Denmark, England, Italy and Poland) (Torp and Adler, 2004; Torp and Nirenberg, 2004; Infantino *et al*., 2007; Lukanowski *et al*., 2008). The basis of morphology is similar to *Fusarium poae* and identified as a "powdery" form of *F. poae*. But the profile of trichothecenes produced is very similar to *Fusarium sporotrichioides* and produced type A trichothecene toxins such as T-2 toxin (Torp and Langseth, 1999; Torp and Nirenberg, 2004). T-2 toxin is highly toxic, inhibit protein synthesis and inducing DNA fragmentation characteristic of apoptosis (Beasley, 1989; Prelusky *et al*., 1994).

The current study has been performed to pursue the following goals: (1) qualitative and quantitative examination of contaminating fungi in stored wheat located in three main wheat producing and storage provinces in Iran including Tehran (north-centre of Iran), East Azarbayejan (region with cold climate in northwest of Iran) and Mazandaran (endemic area of esophageal cancer and region with moderate and humid climate in north of Iran); (2) the presentation of Flotation method (FM) accompanied with Malachite green agar (MGA 0.25) for isolation of toxigenic potential hyaline fungi from grains.

FIG. 1 - Map of Iran, showing relative positions of the three provinces of sampling place (1-3) and provinces related to kind of wheat samples (4-15). 1: East Azarbayejan, 2: Mazandaran, 3: Tehran, 4: Hamadan, 5: Khoozestan, 6: Kordestan, 7: West Azarbayejan, 8: Zanjan, 9: Ardebil, 10: Fars, 11: Ghazvin, 12: Golestan, 13: Kermanshah, 14: Lorestan, 15: Markazi.

MATERIALS AND METHODS

Wheat samples. A total of 109 submitted samples (approximately one kg each sample) of wheat grain were collected from silos and store-pits in the three provinces, Tehran, Mazandaran and East Azarbayejan in the main wheat producing and storage areas in Iran. These samples were related to at least 15 wheat-producing provinces in Iran (Fig. 1, Table 1).

TABLE 1 - List of locations, number and kind of wheat samples collected silos and store-pits in Iran, during 2007

No. of samples	Provinces of related to wheat samples	No. of samples	Provinces of sampling place
13	East Azarbayejan	21	East Azarbayejan
1	Hamadan		
2	Khoozestan		
2	Kordestan		
2	West Azarbayejan		
1	Zanjan		
14	Mazandaran	14	Mazandaran
2	Ardebil	74	Tehran
2	Fars		
3	Ghazvin		
9	Golestan		
6	Hamadan		
3	Kermanshah		
8	Khoozestan		
3	Kordestan		
3	Lorestan		
2	Markazi		
7	Tehran		
2	West Azarbayejan		
1	Zanjan		
2	Mix*(Mar, Ham, Ker)		
2	Mix (Ham, Lor, Kor, W.Az)		
3	Mix (Zan, Lor, Kor, Mar)		
5	Mix (Zan, Kor, Ham, Ard)		
11	Mix		

* Mixed of wheat samples in Iran (Mar: Markazi, Ham: Hamadan, Ker: Kermanshah, Lor: Lorestan, Kor: Kordestan, W.Az: West Azarbayejan, Zan: Zanjan, Ard: Ardebil).

Sampling method. The sampling was done according to International Seed Test Association (ISTA) protocol (Mathur and Kongsdal, 2003). Samples were taken from different horizontal and vertical position chosen at random. Samples were packed in paper bags and immediately sent to the laboratory, where they were stored at 5 ± 1 °C. All of the samples were original cultivated and at least stored for 1 year and intended for human consumption and none had visible signs of mould contamination.

Isolation of fungi.

Flotation method (FM) for isolation of surface mycoflora. Surface contaminating fungal mycoflora from wheat samples without surface disinfection were isolated on the base of FM with MGA 0.25. In this method four hundred kernels (four series, each contains 100 seeds) per subsample were examined. A suspension of 100 kernels in 8 ml sterile distilled water including solutions of Tween 80 (0.005%/ml), penicillin (20000 U/ml) and streptomycin (10 mg/ml), was shaked for 1 min, fixed for 15-30 min and then of the supernatant solution (0.5 ml) was cultured on two plates of MGA 0.25 (Nash-Snyder base containing 0.25 ppm of Malachite Green) by streak method. The plates were incubated in the dark at 28 °C for 4-10 days.

Freeze Blotter (FB) method for isolation of internal mycoflora. For isolation of the internal mycoflora in grains, subsamples of wheat kernels from each sample were surface disinfected in a 1% sodium hypochlorite for one min and rinsed twice in sterile distilled water for 30 s. Four hundred kernels (four series, each contains 100 seeds) per subsample were placed in 25 kernels per plate, according to FB method (Singh *et al*., 1974). The resulting fungal colonies from both methods were recorded and then subcultured in Potato Dextrose agar (PDA).

Identification of fungi. *Aspergillus* species were identified as described by Raper and Fennell (1973), Pitt and Hocking (1997) and Klich (2003). *Fusarium* isolates were purified by single-spore isolation. Single-spore isolates were cultured onto Carnation Leaf agar (CLA), Spezieller Nahrstoffarmer agar (SNA) and PDA plates and incubated at 25-27 °C for 7-14 days under 12 h near UV (nUV) light/12 h darkness cycle (Leslie and Summerell, 2006). Isolates were identified by conidial morphology and colony characteristic as described by Nelson *et al*. (1983) and Leslie and Summerell (2006).

Identification of *Fusarium* cf. *langsethiae*.

Morphological observations. Single-spore isolate was transferred onto PDA, CLA and SNA (with a piece of sterile filter paper placed on the agar surface) to grow at 25 °C with exposure to 12-h alternate cycles of darkness and nUV light (Philips TLD 36W/08 black light, peak 360 nm). All microscopic characters (sporodochial conidia or macroconidia observed under nUV, shape of conidia of aerial mycelium or microconidia globose/napiform, polyphialide and monophialides present, chlamydospore present) and macroscopic characters (powdery appearance on PDA and colony diameter on Potato Sucrose agar (PSA) were according to the Torp and Langseth (1999) and Torp and Nirenberg (2004).

DNA extraction. Fungal DNA was extracted using standard protocols, according to Choi *et al*. (1990) and Rezaie *et al*. (2000) with slight modifications. Briefly, the harvested mycelial mass was flash-frozen in liquid nitrogen and ground to a fine powder in a porcelain mortar. The mycelial powder was suspended in DNA extraction buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM EDTA, 3% SDS and 50 µl of proteinase-K (20 mg/ml). The suspension was then incubated at 65 °C for 1 h and the cellular debris was removed by centrifugation at 2500 *x g* for 15 min. After addition of 25 µl RNase H (10 mg/ml), the suspension was incubated at 37 °C for 30 min, extracted once with phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform-isoamyl alcohol (24:1). The DNA was precipitated by addition of an equal volume of isopropanol, followed by centrifugation at 15000 *x g* for 30 min. Finally, The DNA pellet was rinsed with 70% ethanol and resuspended in distilled water.

PCR and DNA sequencing. DNA of the mentioned *Fusarium* isolate were used in PCR with the *F. sporotrichioides*-specific primers according to Kulik *et al*. (2004) and the *F. langsethiae*-specific primers (Wilson *et al*., 2004). Internal transcribed spacer (ITS) regions of isolate was amplified using universal primers ITS4 and ITS5 (White *et al*., 1990). Amplification was performed in a final volume of 50 µl. Reaction contained 5 µl of 10X PCR buffer, each deoxynucleoside triphosphate (dNTP) at 0.2 mM, each forward and reverse primer at 0.2 µM, 1 µl (50-100 ng) of template DNA, and 5 U of Taq DNA polymerase. The PCR condition consisted of the initial denaturation at 94 °C for 2 min, 35 cycles each of 30 s at 94 °C, 30 s at 58 °C, 1 min at 72 °C, and a final extension at 72 °C for 7 min. The obtained PCR product with an approximate size of 568-bp was sequenced. A 618-646 bp fraction of the Translation elongation factor 1 alpha (TEF-1α) gene was amplified using the primers EF1 and EF2 according to O'Donnell *et al*. (1998) and the protocol described by Carbone *et al*. (1999) with the exception that we used 50 µl PCR mixtures instead of 20 µl. Two external primers (EF1, EF2) (O'Donnell *et al*., 1998) and two nested primers (EF15fwd, EF16rev) (Knutsen *et al*., 2004) were used for reamplification and cycle sequencing. The sequences were aligned using Mega 3, followed by visual inspection and manual adjustment.

The isolation frequency (Fr) and the relative density (RD) of genera and species were calculated according to González *et al*. (2008):

$$\text{Fr (\%)} = \left(\frac{ns}{N}\right) \times 100$$

$$\text{RD (\%)} = \left(\frac{ni}{Ni}\right) \times 100$$

where ns = number of samples where a genus or species of fungi occurred; N = total number of samples; ni = number of isolates of a genus or species; Ni = total number of fungal isolates obtained.

Statistical analysis. The Fisher exact test was applied to analyse possible differences in the isolation frequencies of fungal genera between provinces and applied methods. The analysis was performed by using the GraphPad InStat software V2.02.

RESULTS AND DISCUSSION

Fungi associated with wheat grain

All together, 109 wheat samples were examined. Out of which 681 fungal isolates were recovered on the basis of FM method and 485 fungal isolates were isolated on the basis of FB method. The isolates were belonged to 46 species of 23 fungal genera. Based on Fr, the *Penicillium* spp. (78.4-92.8%), *Aspergillus* spp. (71.4-85.7%) and *Fusarium* spp. (28.6-55.4%) were predominant using FM method. However, dematiaceous fungi such as *Alternaria* spp. (82.4-100%) and *Cladosporium* spp.

(57.1-89.2%) were defected from FB method. The difference was apparent in all three provinces ($P < 0.01$ to $P < 0.0001$). Obviously, the isolating method for identification of *Aspergillus* spp. in east Azarbayejan province and identification of *Fusarium* spp. in Mazandaran province was not significant (Table 2). The exogenous mycoflora of the examined grains has been consisted of *Penicillium* spp., *Aspergillus* spp. and *Fusarium* spp. and the endogenous mycoflora was consisted of *Alternaria* spp. and *Cladosporium* spp. The result of this study is in accordance with other studies (Furlung *et al*., 1995; Weidenboner *et al*., 1997; Krysinska-Traczyk *et al*., 2003; Saberi-Riseh *et al*., 2004). The grains obtained from Mazandaran province were contaminated more by *Aspergillus* spp., *Penicillium* spp. and *Alternaria* spp., but the grains obtained from Tehran and East Azarbayejan were contaminated more with *Fusarium* spp. and *Cladosporium* spp. ($P < 0.05$ to $P < 0.0001$) (Table 2).

According to FM method, among the isolated *Aspergillus* species, the Fr and RD of the *A. niger* (39.4%, 6.3%) and *A. flavus* (36.7%, 5.9%) were more than other species. The significant difference between these two species was not observed ($P > 0.05$). In other words, these two mentioned species were dominant exogenous contaminants of the examined grains. Among these three examined provinces, the abundance of these two species was more in Tehran and East Azarbayejan provinces compared with Mazandaran province ($P < 0.05$ to $P < 0.0001$). According to FB method, the abundance of *A. flavus* (26.6%) and *A. fumigatus* (18.3%) was more than *A. niger* (10.1%). The Fisher exact test method indicated a significant difference between *A. flavus* and *A. niger* ($P < 0.01$). But there was no significant difference between *A. fumigatus* and *A. niger*. *Aspergillus flavus* was the most dominant endogenous *Aspergillus* species contaminating grains. The reports from other countries indicate that the genus *Aspergillus* is the most dominant contaminant of stored wheat and flour and the *A. flavus* is the most abundant *Aspergillus* contaminating wheat and flour (Halt, 1994; Abdullah, 1998; Berghofer *et al*., 2003; Hedayati and Mohammad pour, 2005; Riba *et al*., 2008). The potential risk of *A. flavus* in foodstuff due to aflatoxin production is considerable. Aflatoxin B1 is the most toxic form for mammals and presents mutagenic, carcinogenic and teratogenic properties and has been implicated as causative agent in human hepatic and extrahepatic carcinomas (IARC, 1993). Other *Aspergillus* species that were present at low frequency levels were *A. nidulans*, *A. terreus*, *A. restrictus*, *A. ochraceus*, *A. parasiticus*, *A. candidus* and *A. amstelodami*. Surveys carried out in Iran have shown that natural occurrence of aflatoxins in bread (Hormozdiari *et al*., 1975) as well as ochratoxin A in red wheat (Lacey, 1988).

The Fr and RD of the *Acremonium* species on the basis of FM (58.7%, 9.4%) compared with FB method (1.8%, 0.4%) was highly significant ($P < 0.0001$) (Table 3). Therefore one can present it as exogenous contaminant of grains.

Among the isolated *Fusarium* species (FM method), the Fr and RD of *F. proliferatum* (34%) (5.4%) and *F. verticilloides* (12.8%) (2.3%) was more than other species. The wheat obtained from Tehran province was more contaminated by *F. proliferatum* compared with other provinces ($P < 0.01$) (Table 3). The distribution of this species has implications for human and animal health due to its ability to produce fumonisins, a group of toxic and carcinogenic metabolites that designated as Group 2B carcinogens, that is 'possibly carcinogenic to humans' (Gelderblom *et al*., 1988; IARC, 1993; Jackson *et al*., 1996; Ghiasian *et al*., 2004). The results of the current study are in accordance with the performed study on the field in 23 provinces of Iran (Darvish Nia *et al*., 2006), Golestan province (Zare and Ershad, 1997) and Khozestan province (Moosawi-Jorf *et al*., 2007) and other studies (Lacey, 1988; Saremi and Okhovvat, 2006). However, our results was not in accordance with two other field studies (Zamani-Zadeh and Khorsandi, 1995; Davari *et al*., 2006). In the USA, Canada and Argentina the predominant *Fusarium* species isolated in the field were *F. graminearum* (Walker *et al*., 2001; González *et al*., 2008), while in Europe the most common isolates were *F. graminearum*, *F. culmorum*, *F. avenaceum*, *F. poae* and *F. langsethiae* (Bottalico and Perrone, 2002; Torp and Nirenberg, 2004). Other *Fusarium* species that were present at low frequency levels were *F. subglutinans*, *F. oxysporum*, *F. nygamai, F. culmorum, F. acuminatum*, *F. equiseti*, *F. pseudonygamai*, *F. tricinctum*, *F. lateritium*, *F. chlamydosporum* and *F. babinda*. Studies on fungal species isolated from Iranian cereals have demonstrated their ability to produce trichothecenes (Rezayat *et al*., 1996; Haratian *et al*., 2008), moniliformin (Zamani-zadeh, 1996), fumonisins (Ghiasian *et al*., 2005) and zearalenone (Zamani-Zadeh and Khorsandi, 1995; Saremi and Okhovvat, 2006).

In this study, we have also isolated a rare species known as *F. xylarioides* in Iran. The latter case has been recently reported as Fusarium head blight by Moosawi-Jorf *et al*. (2007).This species is rarely isolated from wheat and may cause coffee wilt disease (Nelson *et al*., 1983).

TABLE 2 - Major fungal genera (hyaline and dematiaceae) recovered from wheat kernels collected of silos and storepits in three provinces in Iran, during 2007

Species	East Azarbayejan				Mazandaran				Tehran				Overall mean			
	FB		FM		FB		FM		FB		FM		FB		FM	
	RD (%)	Fr (%)	RD (%)	Fr (%)	RD (%)	Fr (%)	RD (%)	Fr (%)	RD (%)	Fr (%)	RD (%)	Fr (%)	RD (%)	Fr (%)	RD (%)	Fr (%)
Aspergillus spp.	21.3	66.6	25	71.4	21.7	57.1	30.7	85.7	16.7	50	24.1	73	18.1	54.1	25	74.3
Penicillium spp.	12.3	52.4	14.7	80.9	6.7	28.5	32.3	92.8	12.5	54	18	78.4	11.7	50.4	18.6	80.7
Fusarium spp.	4.5	19	9.5	42.8	10	28.6	9.2	28.6	4.7	14.8	12.3	55.4	5.4	17.4	11.4	49.5
Cladosporium spp.	20.2	85.7	8	52.4	13.3	57.1	3	14.3	25	89.2	11.2	69	22.7	84.4	9.8	58.7
Alternaria spp.	20.2	85.7	5.1	33.3	31.6	100	nd	nd	19	82.4	2.9	18.9	20.8	85.3	3.1	19.2
Total isolates	89		136		60		65		336		480		485		681	
Total samples	21				14				74				109			

FB: Freeze Blotter, FM: Flotation method with MGA 0.25, RD: relative density, Fr: frequency of isolation, nd: not detected.

TABLE 3 - Fungal species recovered from wheat kernels collected of silos and store-pits in three provinces in Iran, during 2007

Species	East Azarbayejan				Mazandaran				Tehran				Overall mean			
	FB		FM		FB		FM		FB		FM		FB		FM	
	RD (%)	Fr (%)	RD (%)	Fr (%)	RD (%)	Fr (%)	RD (%)	Fr (%)	RD (%)	Fr (%)	RD (%)	Fr (%)	RD (%)	Fr (%)	RD (%)	Fr (%)
Absidia spp.	2.2	9.5	2.2	14.3	nd	nd	1.5	7.1	nd	nd	1	6.7	0.4	1.8	1.3	8.2
Acremonium spp.	2.2	9.5	8.8	47.6	nd	nd	6.1	28.5	2	9.4	10	64.8	1.8	8.2	9.4	58.7
Alternaria spp.	20.2	85.7	5.1	33.3	31.6	100	nd	nd	19	82.4	2.9	18.9	20.8	85.3	3.1	19.2
Aspergillus amstelodami	nd	nd	nd	nd	nd	nd	nd	nd	0.3	1.3	0.2	1.3	0.2	0.9	0.1	0.9
Aspergillus candidus	nd	nd	nd	nd	nd	nd	nd	nd	0.3	1.3	0.2	1.3	0.2	0.9	0.1	0.9
Aspergillus flavus	7.8	33.3	6.6	42.8	10	42.8	4.6	21.4	4.7	21.6	5.8	37.8	6	26.6	5.9	36.7
Aspergillus fumigatus	5.6	23.8	6.6	42.8	1.6	7.1	4.6	21.4	4.2	18.9	3.7	24.3	4.1	18.3	4.4	27.5
Aspergillus nidulans	nd	nd	0.7	4.7	nd	nd	3	14.3	nd	nd	1.9	12.1	nd	nd	1.8	11
Aspergillus niger	4.5	19	6.6	42.8	nd	nd	3	14.3	2	9.4	6.7	43.2	2.3	10.1	6.3	39.4
Aspergillus ochraceus	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.2	1.3	nd	nd	0.1	0.9
Aspergillus parasiticus	nd	nd	nd	nd	nd	nd	nd	nd	0.3	1.3	0.2	1.3	0.2	0.9	0.1	0.9
Aspergillus restrictus	nd	nd	nd	nd	nd	nd	nd	nd	0.6	2.7	nd	nd	0.4	1.8	nd	nd
Aspergillus terreus	nd	nd	nd	nd	nd	nd	1.5	7.1	1.2	5.4	1.6	10.8	0.8	3.7	1.3	8.2
Aspergillus spp.	3.4	14.3	4.4	23.8	10	35.7	13.8	35.7	3	13.5	3.5	22.9	3.9	17.4	4.7	31.2
Aureobasidium pullulans	4.5	19	0.7	4.7	nd	nd	nd	nd	nd	nd	nd	nd	0.8	3.7	0.1	0.9
Bipolaris spp.	1.1	4.7	nd	nd	1.6	7.1	nd	nd	1.2	5.4	0.2	1.3	1.2	5.5	0.1	0.9
Cladosporium spp.	20.2	85.7	8	52.4	13.3	57.1	3	14.3	25	89.2	11.2	69	22.7	84.4	9.8	58.7
Curvularia spp.	nd	nd	nd	nd	1.6	7.1	nd	nd	0.3	1.3	nd	nd	0.4	1.8	nd	nd
Epicoccum spp.	nd	nd	nd	nd	nd	nd	1.5	7.1	nd	nd	0.2	1.3	nd	nd	0.3	1.8
Fusarium acuminatum	1.1	4.7	0.7	4.7	nd	nd	nd	nd	nd	nd	nd	nd	0.2	0.9	0.1	0.9
Fusarium babinda	nd	nd	nd	nd	nd	nd	nd	nd	0.3	1.3	0.2	1.3	0.2	0.9	nd	nd
Fusarium chlamydosporum	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.2	1.3	nd	nd	0.1	0.9
Fusarium culmorum	nd	nd	nd	nd	1.6	7.1	nd	nd	0.3	1.3	nd	nd	0.4	1.8	nd	nd
Fusarium equiseti	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.2	1.3	nd	nd	0.1	0.9
Fusarium cf. *langsethiae*	nd	nd	nd	nd	nd	nd	nd	nd	0.3	1.3	nd	nd	0.2	0.9	nd	nd
Fusarium lateritium	nd	nd	0.7	4.7	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.1	0.9
Fusarium nygamai	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	1.2	8.1	nd	nd	0.9	5.5
Fusarium oxysporum	1.1	4.7	0.7	4.7	nd	nd	nd	nd	0.3	1.3	0.6	4	0.4	1.8	0.6	3.7
Fusarium proliferatum	nd	nd	2.9	19	3.3	14.3	4.6	21.4	1.8	8.1	6.2	40.5	1.6	7.3	5.4	34
Fusarium pseudonygamai	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.2	1.3	nd	nd	0.1	0.9
Fusarium subglutinans	nd	nd	0.7	4.7	nd	nd	1.5	7.1	0.9	4	1.6	10.8	0.6	2.7	1.5	9.2
Fusarium tricinctum	nd	nd	nd	nd	nd	nd	nd	nd	0.3	1.3	nd	nd	0.2	0.9	nd	nd
Fusarium verticillioides	2.2	9.5	2.9	19	1.6	7.1	3	14.3	0.3	1.3	1.6	10.8	0.8	3.7	2.3	12.8
Fusarium xylarioides	nd	nd	0.7	4.7	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.1	0.9
Fusarium spp.	nd	nd	nd	nd	3.3	14.3	nd	nd	0.3	1.3	0.2	1.3	0.6	2.7	0.1	0.9
Mucor spp.	3.4	14.3	0.7	4.7	nd	nd	1.5	7.1	1.5	6.7	2.1	13.5	1.2	7.3	1.8	11
Nigrospora spp.	nd	nd	nd	nd	nd	nd	nd	nd	0.3	1.3	nd	nd	0.2	0.9	nd	nd
Paecilomyces spp.	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.2	1.3	nd	nd	0.1	0.9
Penicillium spp.	12.3	52.4	14.7	80.9	6.7	28.5	32.3	92.8	12.5	54	18	78.4	11.7	50.4	18.6	80.7
Rhizomucor spp.	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.2	1.3	nd	nd	0.1	0.9
Rhizopus spp.	3.4	14.3	3.7	23.8	nd	nd	7.7	35.7	2.7	12.1	6.4	41.8	2.5	11	6	37.6
Scopulariopsis spp.	nd	nd	0.7	4.7	1.6	7.1	nd	nd	nd	nd	1.2	8.1	0.2	0.9	1	6.4
Stemphillium spp.	nd	nd	nd	nd	nd	nd	nd	nd	0.3	1.3	0.2	1.3	0.2	0.9	0.1	0.9
Syncephalastrum spp.	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.4	2.7	nd	nd	0.3	1.8
Torula spp.	nd	nd	nd	nd	nd	nd	nd	nd	0.3	1.3	nd	nd	0.2	0.9	nd	nd
Trichoderma spp.	1.1	4.7	nd	nd	nd	nd	3	14.3	nd	nd	nd	nd	0.2	0.9	0.3	1.8
Trichothecium spp.	1.1	4.7	0.7	4.7	nd	nd	nd	nd	nd	nd	nd	nd	0.2	0.9	0.1	0.9
Ulocladium spp.	1.1	4.7	0.7	4.7	10	42.8	nd	nd	6.5	29.7	0.2	1.3	6	26.6	0.3	1.8
Yeast spp.	1.1	4.7	11	61.9	nd	nd	nd	nd	2.4	10.8	5.8	37.8	1.8	8.2	6.3	37.6
Un known spp.	nd	nd	8	33.3	1.6	7.1	3	14.3	4.5	20.3	2.9	18.9	3.3	14.7	4	21.1
Total isolates	89		136		60		65		336		480		485		681	
Total samples		21				14				74				109		

FB: Freeze Blotter, FM: Flotation method with MGA 0.25, RD: relative density, Fr: frequency of isolation, nd: not detected.

Description of *Fusarium* cf. *langsethiae*

Macroscopic observation

In primary culturing on PDA medium, they appeared as pink colour, had slow growth and possessed small air mycelium. On repeated passage on PDA medium they appeared as white coloured with powdered characteristic. On PSA medium they demonstrated a rapid growth and possessed more air mycelium. Radial growth rate on PSA and PDA at 25 °C in complete darkness was 8.5 and 7.5 mm/day, respectively, at 20 ± 0.5 °C on PDA, 6.4 mm/day.

Microscopic observation

Conidia napiform or globose, nonseptate, measuring: 5.1-9.4 x 3.8-6.4 µm with average and S.D. of 6.7 ± 1 x 5 ± 0.6 µm. Chlamydospores not formed, no sporodochial and macroconidia under nUV, conidiophores short and branched, branched monophialides, polyphialide, and ampuliform phialides (Fig. 2).

Molecular properties

The sequencing result of PCR product that was based on universal primers ITS4 and ITS5 (568 bp) with two registered records

in GenBank/EMBL database belonged to *F. langsethiae* with accession number EF526078 and AY680864. This result indicated 100% identity. The isolated species did not respond to specific primers belonging to *F. sporotrichioides* as Kulik *et al*. (2004) but did respond to specific primers belonging to *F. sporotrichioides* that designed by Wilson *et al*. (2004). The specific primers belonging to *F. langsethiae* that designed by these authors did not any positive PCR. The sequencing result of PCR product that was based on external primers (EF1, EF2) and nested primers (EF15, EF16) for TEF-1α gene with registered sequences recorded in GenBank/EMBL database related to *F. langsethiae* (accession Nr. AJ427272), revealed high homology with the same genes from *F. langsethiae* (99%). However, the same results have been detected by homology search with *F. sporotrichioides* species (accession Nr. EF521146, AY337442, AJ420840, AJ420820) as well as 11 other *F. sporotrichioides* species recorded. With regard to the morphological results, ITS1, ITS2 and TEF-1α gene sequences, this species is similar to *F. langsethiae* (IBT 9959). On the basis of morphology, Kristensen *et al*. (2005) has classified the IBT 9959 species as *F.* cf. *langsethiae,* however on the basis of TEF-1α gene it is classified as *F. sporotrichioides*. Thus this isolate could be considered as the intermediat species of *F. langsethiae* and *F. sporotrichioides*. This isolated species has been discovered for first time in Iran. According our knowledge it has not been reported so far such case in Asia. This is the second report in the literature.

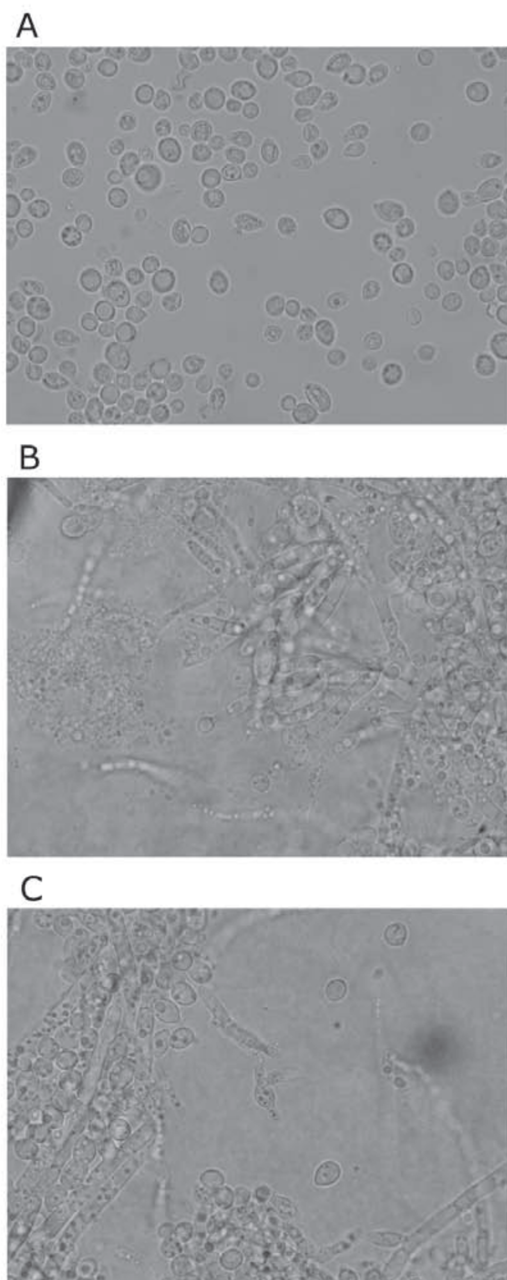

FIG. 2 - *Fusarium* cf. *langsethiae* on Spezieller Nahrstoffarmer agar at 25 °C after 14 days under nUV. A: napiform/globose microconidia, 100X; B: branched monophialides, 100X; C: ampuliform phialides, 100X.

With regard to our experience in using the flotation method on soil for isolation of the potentially toxigenic species including *Penicillium* spp., *Aspergillus* spp., and *Fusarium* spp. as dominant species, we applied this method by some modification for isolation of fungal exogenous species from grains. In this method instead of using MGA 2.5 that introduced by Castella *et al*. (1997a, 1997b) and Bragulat *et al*. (2004) for isolation of *Fusarium* species specially *F. verticilloides*, we used of MGA 0.25 ppm for isolation of other fungi specially *Fusarium* spp. and *Aspergillus* spp. that also mucoral fungi growth restricted.

CONCLUSIONS

With regard to our results by application of FM method, we can recommend this method for isolation of toxigenic potential hyaline fungi such as *Penicillium* spp., *Aspergillus* spp., *Fusarium* spp. and *Acremonium* spp. from grains. In most cases, moreover, this method can observe only one colony on MGA 0.25 ppm medium.

Fusarium langsethiae and *F. sporotrichioides* are known to produce the T-2 toxin (the most toxin of type A trichotecenes) which is related to alimentary toxic aleukia in human. Therefore, a large-scale monitoring of these fungi and T-2 toxin on wheat is necessary. The high frequency of *Alternaria* and *Cladosporium* species as the endogenous mycoflora of stored wheat, emphasizes the importance of next research on those toxins in Iranian stored wheat.

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