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Natural Occurrence of *Fusarium* species in Maize Kernels at Gholestan Province in Northern Iran

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Abstract: *Fusarium* species in 41 samples of maize, which related to pre-harvest time, harvest time, enter to silo and exit from silo that collected in 2004 from North of Iran at Gholestan province were isolated and identified. For isolation the *Fusarium* species, maize were cultured onto Nash-snyder agar, then preparing a single spore and sub cultured them onto Potato Dextrose Agar (PDA) and Spezieller Nahrstoffarmer Agar (SNA) mediums. The most prevalent *Fusarium* species isolated were *Fusarium verticillioides*, followed by *F. proliferatum*, *F. oxysporum*, *F. culmorum*, *F. solani*, *F. equiseti* and *F. poae*. The results of this work showed high incidence of *F. verticillioides* and *F. proliferatum* in maize kernels that was in accordance with the other studies in Iran and other countries.

Key words: *Fusarium*, Gholestan, Iran, maize

INTRODUCTION

Maize (*Zea mays* L.) is one of the four major foods of the world population and is the crop that has the first position in production level in the world (Anderson *et al*., 2004). Also maize is one of the most prevalent grains contaminated by *Fusarium* (Fandohan *et al*., 2003) and worldwide reports has been shown that most of *Fusarium* contamination on crops in all over the world, have been conducted mostly on this grain. *Fusarium* is a phytopathogenic fungus with a global distribution and is found in a variety of agricultural products generally on maize, wheat and other cereal grains being consumed by human and animal. Because of reduction of quality and quantity in the agricultural crops, *Fusarium* contamination is a major problem in the world (Jurado *et al*., 2006). *Fusarium* with a range of various taxa that have different ecological, morphological and physiological characteristics is a heterogeneous fungal genus. However cosmopolitan and often persistent in plant, or plant residues, soil and organic matter, variations in fungal community structure and diversity could be related with special climatic areas of the world. In some ecological localities, a great number of minor species might play significant roles in the infection process competing with pathogenic fungi in plants or in soil (Vujanovic *et al*., 2006). The most important climatic factors, which influence the improvement of *Fusarium* diseases of cereals are temperature and humidity, while the effect of these climatic factors is not independent of other environmental and host factors (Doohan *et al*., 2003; Brennan *et al*., 2003). *Fusarium* species causes two types of maize ear rot called red ear rot and pink ear rot, which mostly caused by species of the *Discolour* section and the representatives of the *Liseola* section, respectively. *F. graminearum*, *F. culmorum*, *F. cerealis* and *F. avenaceum* are the main species causing maize red ear rot. *F. verticillioides*, *F. proliferatum* and *F. subglutinans* are the species frequently isolated from maize pink ear rot. *F. equiseti*, *F. poae*, *F. sporotrichioides*, *F. acuminatum*, *F. semitectum*, *F. solani* and *F. oxysporum* are the other toxigenic *Fusarium* species less frequently isolated from both types of maize ear rot (Logrieco *et al*., 2002). According to the worldwide reports, *F. verticillioides*, *F. proliferatum* and *F. subglutinans* are the main agents that responsible for ear rot. Infection can be seedborne and systemic in the crop from seedling to harvest, or starting during the pollination where the silks are infected by the airborne conidia. During harvest, ear rot seems as individual rotted kernels or as arbitrarily scattered groups of rotted kernels (White, 2000; Morales-Rodriguez *et al*., 2007). It is important to isolate and identify the *Fusarium* species because they are well known to produce a wide variety of mycotoxins which causes infection diseases in human and animal that consuming them. A varied range of

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mycotoxins such as fumonisins, moniliformin (MON), trichothecenes, zearalenol (ZEN), beauvericin (BEA) and enniatins (Ens) produces by *Fusarium* species (Rheeder *et al*., 2002; Llorens *et al*., 2006). Different *Fusarium* species or strains with various toxigenic potential may produce several mycotoxins in processed grains that cause the infection of the grain. (Bottalico and Perrone, 2002). Cereals are usually used in feed and therefore farm animals will use, relatively high amounts of mycotoxins. It is with diagnostic value if we identify the fungi in case of mycotoxins epidemics. However, definite conclusion is possible just with tracing mycotoxins. Some of *Fusarium* species are not stable in culture and they mutate. The mutants may be either the mycelial or pionnotal type and often lose virulence and the ability to produce toxins (Nelson *et al*., 1983). Whilst *Fusarium* is grown on a medium rich in carbohydrates, cultural mutations often occur (Nelson, 1992). The most common *Fusarium* species found in affected grain in Iran's maize kernel is *F. verticillioides* (called before *F. moniliforme*) (Ghiasian *et al*., 2004). Fumonisins are the important mycotoxins occurred broadly in Iran's maize (Ghiasian *et al*., 2005). *Fusarium* species are probably one of the most important toxigenic fungi in northern Iran because of climatic condition with dominant species which depend on the area and the type of crop infected. Because maize is one of the most important cereals produced and imported in Iran the object of this study was assigned to identify the main *Fusarium* species in maize grain in the areas in North of Iran.

MATERIALS AND METHODS

Maize sampling: A total of 41 (5-10 kg) samples, related to pre harvest time, harvest time, enter to silo and exit from silo were collected in the areas of Gholestan province from North of Iran which is located in the endemic esophageal cancer (OC) area in 2004, mostly intended for animal and some for human consumption without indicating noticeable signs of mold contamination.

Isolation of *Fusarium*: All grain from each sample were outside disinfected for 1 min with a 5% NaOCl solution, washed two times in sterile distilled water and dried in a laminar flow cabinet. Then all samples were grounded using a Romer analytical mill (Union, MO, USA) and one-third selected for isolation *Fusarium* species. Isolate were made in two ways as described: (1) grounded maize were plated onto Peptone PCNB (Pentachloronitro benzene) agar or Nash-Snyder agar in three plates (Nash and Snyder, 1962) modified by Nelson *et al*. (1983) and (2) 25 g of grounded samples was added to 200 mL deionoised water while shook at 140 rpm for 30 min then 1 mL of each was placed into the same medium in three plates. This medium contained 1000 mg L^{-1} of streptomycin sulfate, to suppress the growth of bacteria. Pentachloronitrobenzene (PCNB) cause reduction in growth of fast growing fungi including Zygomycetes such as Mucor and Rhizopus and prevent their extra ordinary generation and their colony mixing (Singleton *et al*., 1992). The plates were incubated for 5-7 days at 25-27°C in the dark. With observing the colonies, prepared a single spore of them.

Single spore isolation: This method was used to gain unmixed cultures, divide species in mixed cultures and to permit for uniform and consistent production of conidia (Singleton *et al*., 1992). Therefore because some colonies with close vicinity may mixed cultures, so isolates were purified by preparing single-spore cultures as follows: by scraping conidia from PDA (Phtato dextrose agar) a suspension of conidia in 10 mL sterile distilled water was prepared to obtain a concentration of 1-10 conidia in a drop viewed under the low power objective (10x) of a microscope. A droplet of suspension was splashed on the PDA plates and incubated at 25-27°C for 24 h. After verification of plates with stereomicroscope and observing the single germinated conidia, it was removed along with small piece of agar and transferred to PDA slants.

Identification of *Fusarium* species: For identification of *Fusarium* species, subcultures were transferred to PDA (potato dextrose agar) and SNA (Spezieller Nahrstoffarmer Agar) respectively for macroscopic and microscopic identification and incubated for 5-7 days at 25-27°C. In case along time passes from *Fusarium* culture or, *Fusarium* are several times subcultured, the mutates may occurs. To identify the species macroscopic specifications such as color, type of growth of mycelium; and microscopic specifications such as type of phialides (monophialides or polyphialides), presence or absence of microconidia chain, false heads, presence or absence and shape of macroconidia, presence or absence of chlamydospores was investigated. The identification of *Fusarium* species was made upon Nelson *et al*. (1983).

The isolation Frequencies (Fq) and Relative density (Rd) of species isolated were calculated as follows:

$$\text{Frequency}(\%) = \frac{\text{No. of samples in which a species occurred}}{\text{Total No. of samples}} \times 100$$

$$\text{Relative density}(\%) = \frac{\text{No. of isolates of a species}}{\text{Total No. of species isolates}} \times 100$$

RESULTS AND DISCUSSION

In the present study the *Fusarium* species in 41 maize samples that related to pre-harvest time, harvest time, enter to silo and exit from silo, which collected in 2004 from North of Iran at Gholestan province were investigated. *Fusarium* species were identified according to the Nelson *et al.* (1983). From the total samples, 1008 *Fusarium* species were isolated. *F. verticillioides* (with 60.41% of total isolates) was the most predominant species, followed by *F. proliferatum* (13.39%), *F. oxysporum* (7.44%), *F. culmorum* (5.64%), *F. solani* (4.17%), *F. equiseti* (1.48%), *F. poae* (1.19%) and other species with lower incidence (5.96%) (Table 1). Occurrence of *F. verticillioides* in harvest stage with frequency of 41.46% and relative density of 30.35% was the highest amount, compared to other stages. *F. proliferatum* was also the highest amount in harvest stage weighed against other stages with frequency of 21.95% and relative density of 6.55%. Occurrence of *F. oxysporum*, *F. culmorum* and *F. solani* had also the highest statistics during harvest stage in comparison to other stages with frequency of 14.63, 12.19, 7.31% and relative density of 3.57, 2.38 and 1.78%, respectively. But *F. equiseti* and *F. poae* were the most prevalent in exit from silo stage with frequency and relative density of 4.87 and 0.69% (for *F. equiseti*) and 4.87 and 87% (for *F. poae*), respectively in compare with other stages (Table 2). Frequency of *F. verticillioides* in enter to silo stage comparing to the same stage was 100% that indicate this fungus has been existed in all the samples in this stage (Table 3). The result of this study showed that, 60.41% of the species was *F. verticillioides*, which demonstrated the high frequency of this seed-born species in maize. Similar studies also found that *F. verticillioides* has the same prevalence in other countries such as Brazil (Rodriguez and Sabino, 2002) USA (Jurjevic *et al.*, 2004), Nigeria (Bankole *et al.*, 2003; Bankole and Mabekoje, 2004; Timothy *et al.*, 2007) Colombia (Acuna *et al.*, 2005) and Spain (Jurado *et al.*, 2006). *F. proliferatum* is the second most predominant *Fusarium* species with frequency and relative density of 46.34 and 13.39%, respectively in this study. These results are in accordance with other results in Iran and other countries (Zare and Ershad, 1997; Srobarova1 *et al.*, 2002; Ghiasian *et al.*, 2004; Acuna *et al.*, 2005). Presence of *F. verticillioides* and *F. proliferatum* were found in earlier studies in northern Iran as the most common seed-borne fungi on farm rice, maize and wheat kernels (Zare and Ershad, 1997). Concerning locality, it was demonstrated that, incidence of *F. verticillioides* was much higher in Mazandaran province in compare with other areas in Iran including Khuzestan and Kermanshah provinces, from field-testing results gained on the fungal mycoflora research made in 2000, regarding to maize kernels in the main maize producing areas (Ghiasian *et al.*, 2004). Another study about maize has shown that *F. verticillioides* and *F. proliferatum* were the most major species isolated from maize, after *Aspergillus* species, in the northern parts of Iran (Boujari and Ershad, 1995). Also *F. verticillioides* with 92% and *F. proliferatum* with 8% incidence was isolated from maize in North of Iran and Karaj town in Iran (Zamani and Alizadeh, 2000). In spite of Norred (1993) study, which indicated the incidence of *F. verticillioides* in OC (Oesophaseal cancer area surrounding North of Iran, is low, the other studies, which mentioned above and the present work indicate the most incidences of *F. verticillioides* in these areas. The occurrence of *Fusarium* species in the maize samples that recovered in our study agreed with surveys carried out worldwide where *F. verticillioides*, *F. proliferatum* and *F. graminearum* are the most frequently isolated species in maize and *F. equiseti*, *F. poae*, *F. sporotrichioides* and *F. culmorum* are considered less frequent (Logrieco *et al.*, 2002). The difference between their study and the present study is that in our study no *F. graminearum* and *F. sporotrichioides* isolated instead we isolate *F. solani* and *F. oxysporum.* In 2007 in Mexico in maize through morphological and phylogenetic analysis, seven species of *Fusarium* identified as *F. chlamydosporum*, *F. napiforme*, *F. poae*, *F. pseudonygamai*, *F. solani*, *F. subglutinans* and *F. verticillioides*, were found to be associated with ear rot disease and *F. chlamydosporum*, *F. poae*, *F. pseudonygamai*, *F. subglutinans* and *F. verticillioides* were found within asymptomatic kernels as well (Morales-Rodriguez *et al.*, 2007). Not only high incidence of *F. verticillioides* demonstrates its importance as a seed-borne fungus, often causing symptomless infections in maize grains, but also it is because of its potential toxigenicity. *F. verticillioides* and *F. proliferatum*, the main species isolated in the present study, are the most common fumonisin producing fungi associated with corn (Marasas *et al.*, 2001). The spread of

Table 1: Frequency, relative density of *Fusarium* species in maize from Gholestan provinces in northern Iran

Fusarium species	Frequencies (%)	Relative density (%)
F. verticillioides	87.80	60.41
F. proliferatum	46.34	13.39
F. oxysporum	29.26	7.44
F. culmorum	21.95	5.65
F. solani	24.39	4.17
F. equiseti	9.75	1.48
F. poae	9.75	1.19
Other species	19.51	5.96

Table 2: Frequency and relative density of *Fusarium* species in maize derived from four collected stages in compare with whole sampling stages in Gholestan province

	Pre-harvest time		Harvest time		Enter to silo		Exit from silo	
Fusarium species	Fq (%)	RD (%)	Fq (%)	RD (%)	Fq (%)	RD (%)	Fq (%)	RD (%)
F. verticillioides	12.19	8.93	41.46	30.35	19.51	11.61	14.63	9.82
F. proliferatum	7.32	2.38	21.95	6.55	7.32	3.57	9.75	0.89
F. oxysporum	4.88	0.30	14.63	3.57	ND	ND	9.75	3.57
F. culmorum	ND	ND	12.19	2.38	2.43	1.18	7.31	2.08
F. solani	4.87	0.89	7.32	1.78	7.32	0.89	4.87	0.59
F. equiseti	ND	ND	2.44	0.30	2.44	0.49	4.87	0.69
F. poae	4.87	0.30	ND	ND	ND	ND	4.87	0.87
Other *Fusarium* species	2.44	1.19	7.32	2.98	9.75	1.79	ND	ND

Table 3: Frequency and relative density of *Fusarium* species in maize derived from each of four collected stages comparing to the same stage in Gholestan province

	Pre-harvest time		Harvest time		Enter to silo		Exit from silo	
Fusarium species	Fq (%)	RD (%)	Fq (%)	RD (%)	Fq (%)	RD (%)	Fq (%)	RD (%)
F. verticillioides	83.33	63.83	89.47	63.35	100.00	59.09	75.00	53.22
F. proliferatum	50.00	17.02	47.37	13.66	37.50	18.18	50.00	4.83
F. oxysporum	33.33	2.13	31.58	7.45	ND	ND	50.00	19.35
F. culmorum	ND	ND	26.31	4.96	12.50	6.06	37.50	11.29
F. solani	33.33	6.38	15.79	3.72	37.50	4.54	25.00	3.22
F. equiseti	ND	ND	5.26	0.62	12.5	2.53	25.00	3.76
F. poae	33.33	2.12	ND	ND	ND	ND	25.00	4.83
Other *Fusarium* species	16.66	8.51	15.79	6.21	50	9.09	ND	ND

this species has harmful influence on human and animal health because of its capability to generate fumonisins which is a group of toxic and carcinogenic metabolites (Ghiasian *et al*., 2005). International Agency for Research on Cancer (IARC) has also labeled fumonisins as Group 2B carcinogens, possibly carcinogenic to humans (IARC, 1993). *F. oxysporum* produces toxins such as Diacetoxyscirpenol and T-2 toxin. *F. culmorum* generates B trichothecene, especially deoxynivalenol which is harm for human in case exceeding 1000 ng g^{-1}. *F. solani* causes kratitis and is known as an opportunistic pathogen in human and animals. Also it produces toxins like HT-2 toxin and Neosolaniol. *F. equiseti* produces toxins such as Equisetin, Diacetoxyscirpenol and nivalenol and at last *F. poae* generates type A of trichothecene such as T-2 toxin and enniatins and beauvericin (Marasas *et al*., 1984; Morrison *et al*., 2002; Logrieco *et al*., 2002). *F. verticillioides* (called before *F. moniliforme*) is one of the most prevalent fungi which are related to main human and animal nutritional samples such as maize. Due to early definition of this fungus, it was found suspicious of being involved in human and animal diseases. *F. moniliforme* is in the section *Liseola* along with *F. proliferatum*, *F. subglutinans* and *F. anthophilum*. *F. moniliforme* is the reason for ear rot and stalk rot of maize and it is widely infecting the maize kernels. Maize kernels may get infected through the silks, through holes and fissures in the pericarp or at points where the pericarp scratched by the emerging seedling and as a result of systemic infection of the maize plant by *F. moniliforme* (Nelson, 1992). Temperature and osmotic stress are the most important factors that influence on host susceptibility to fungal disease (Conrath *et al*., 2002). It is possible that *Fusarium* spp. populations vary within and between harvesting seasons (Bateman and Murray, 2001). Among fungal genera that contaminate maize, *F. verticillioides* and *F. proliferatum* are dominant over a wide range of temperature and a_w conditions (Doohan *et al*., 2003). Values of Topt and Tmax for *F. verticilloides* were set at 31 and 35°C, respectively corresponding with evidence that indicated *F. verticillioides* grow faster at higher temperatures (Reid *et al*., 1999). Production of *F. verticillioides* occurs during higher temperatures and drier years compared to warm and dry conditions of the subtropics and dry land maize (Vigier *et al*., 1997). *F. verticillioides*, the causal agent of *Fusarium* ear rot, produces a whitish-colored mold growth that tends to be scattered on the ear (Reid *et al*., 1999). *Fusarium* species contaminating maize kernels are competing with other fungi including the other *Fusarium* species and competition between these fungi can have clear influence on final concentration of mycotoxins. *F. verticillioides* suppresses the growth of other maize ear fungi including *F. graminearum* and *Aspergillus flavus*. Evidences indicate that *F. verticillioides* has the higher ability of colonization rather than other *Fusarium* species in maize (Stewart *et al*., 2002). In this study among all samples, occurrence of *F. verticillioides* was the most prevalent with frequency and relative density of 87.8 and 60.41% followed by *F. proliferatum* with frequency and relative density of 46.34 and 13.39%, respectively. After these two species *F. oxysporum*, *F. solani* and *F. culmorum* graded highest occurrence sequenced with frequencies of 29.26, 24.39 and 21.95% followed by *F. equiseti* and *F. poae* with frequency of 9.75% for each case (Table 1). Eventually, the results of this study indicate the high incidence of *F. verticillioides* followed by *F. proliferatum* resulting from the temperature, that is dominant in North of Iran like Gholestan province.

CONCLUSION

This study expresses high occurrence of *F. verticillioides* with frequency and relative density of 87.8 and 60.41% followed by *F. proliferatum* with frequency and relative density of 46.34 and 13.39%, respectively which indicate prevalence of these two species in Iranian maize kernels especially in wet area.

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