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DETECTION OF AFLATOXIN CONTAMINATION AND INCIDENCE OF FUNGI ASSOCIATED WITH RED CHILI AVAILABLE IN LOCAL MARKET OF KARACHI, PAKISTAN

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Abstract

Chili (*Capsicum annuum* L.), a valuable cash crop of Pakistan is cultivated for both local consumption as well as for export. However, its export is being affected by aflatoxin contamination. Many chili consignments have been found to have aflatoxin concentration greater than the allowable limit. In this study, different chili samples were collected from local markets as well as from chili fields for the detection of aflatoxins and estimation of the incidence of associated fungi with chili fruit. Aflatoxin (AF) contamination was determined by Enzyme-Linked Immunosorbent Assay (ELISA) and it was found between the range of 3.2 to 39.2 ppb. However, aflatoxin was found less than 10 ppb in most of the samples. The highest incidence of *Aspergillus niger* was found in all twenty-one test samples followed by *Penicillium* spp., *Aspergillus flavus, Trichoderma* spp., and *Rhizopus* sp. This investigation showed that the export of Pakistani chili is not at the stack, since more than 50% of chili samples showed the presence of aflatoxin within the acceptable range for human consumption as per US Food and Drug Administration (FDA) standard.

Key words: Aflatoxin, Fungi, *Aspergillus flavus*, Red chili, Pakistan.

Introduction

Red chili (*Capsicum annuum* L) is the major part of Asian cuisine and is widely used in traditional and continental food as a major ingredient of spices (Mushtaq *et al*., 1997; Saleh *et al*., 2018). Chili is also used in the pharmaceutical and cosmetic industries (Kuchi *et al.*, 2014). Pakistan ranked 4th in chili production worldwide with an average production of 2.27 tonne per ha., where different varieties of chili are grown mainly in lower regions of Sindh province including Kunri, Umerkot, and Mirpurkhas (Khan *et al.,* 2020). Dandi cut or Longi is the dominant variety, famous for its color and pungency, and it is grown for export purposes, where Sindh contributes 85-88% of total chili production of Pakistan (Anon., 2010; 2017). However, improper postharvest handling affects its quality via fungal contamination and aflatoxin production which ultimately affects its export (Sahar *et al.*, 2013; Paterson, 2007). Chili is transported and stored in jute bags, where improper logistic practices may be responsible for damaging the quality of pods (Iqbal *et al.*, 2010), and with increasing its storage-period risk of fungal growth and aflatoxin production also increases (Iqbal *et al.*, 2011). It has been estimated that Pakistan produces a total 90,000 tons of red chili, (Anon., 2010), however rejection of chili consignment and the restrictions imposed have acted as an invisible trade barrier. The current study was designed to investigate the extent of fungal association and aflatoxin contamination in red chili var., longi grown in different areas of Pakistan for local consumption and export purposes, since aflatoxin contamination has been posing a serious problem in the international trade of chilies (Sudha *et al*., 2009).

Material and Method

Study design and sample collection: Twenty-one chili samples were collected from the local markets belongs to different areas of Pakistan, were categorized as:

1- Unpacked unground chili pod (whole)
2- Unpacked ground (powder) chili
3- Packed ground (powder) - commercial

Chili samples were collected from the "Jordia bazaar" (main trading center of Karachi, Pakistan) and grocery stores. Chili variety longi of different origin (Kunri, Sakro, Moro, Quetta, Sargodha) were purchased, besides some other varieties. Samples were collected randomly, tagged with date of sampling, name of variety, and locality. Whereas, packed chili ground (powder) sold by the local/ national companies in packing of 100g, 200g, 500g, and 1000g were collected from small and large superstores within the vicinity of Karachi.

Isolation of fungi from chili samples: For quantitative evaluation of fungal incidence, chili samples were grinded in a sterile electric grinder and serial dilution was made up to 1:100 in sterilized distilled water. From final dilution 100 µL was poured onto the Potato Dextrose Agar (PDA) plates amended with penicillin (100000 units/mL) and streptomycin (0.2 g/mL). Each sample had three replicates and plates were incubated for 7 days at 28 °C. Isolated fungal colonies were identified with reference to Barnett & Hunter (1998); Booth (1971); Domsch *et al*., (1980), Dugan (2006), Ellis (1971); Nelson *et al*., (1983); Raper & Fennel (1965) and Raper & Thom (1949).

Fungal cultures were maintained on PDA slants and the frequency of occurrence of each fungus was calculated using the formula:

$$\% \text{ Occurrence of a fungus} = \frac{\text{No. of occurrences of particular fungus}}{\text{Total no. of sample examined}} \times 100$$

The population of each fungus was also calculated by using the formula:

$$CFUg^{-1} = \text{Number of fungal colonies on plate} \times \text{Dilution factor}$$

Samples preparation for total aflatoxin: For the estimation of total aflatoxin, a 10g ground (powder) chili sample was homogenized in 50 mL HPLC grade methanol (70%) and filtered through Whatman filter paper No. 1. The filtrate was used for the aflatoxin estimation (Murshed *et al.*, 2019).

Quantitative analysis of total aflatoxin: Total aflatoxin contamination was quantitatively determined by using Enzyme-linked Immuno-sorbent Assay (ELISA), using Veratox kit (Neogen, Lansing, Michigan, USA) as per manufacturer's instruction. Absorbance was recorded on a microplate reader (iMark, BioRad, USA) at 650 nm against aflatoxin standard (Okuma *et al*., 2018, Sahar *et al*., 2015).

Result and Discussion

Warm temperature and high humidity provide a conducive environment for the growth of fungi at pre and post-harvest stages (Paterson, 2007; Costa *et al.*, 2019). Chilies are susceptible to infection by aflatoxin producing fungi and subsequent contamination by aflatoxins at every stage (Singh & Cotty, 2017). In the current study, it was observed that fungal communities associated with chilies cultivated in different regions of Pakistan belong to Ascomycota and Basidiomycota. These isolated fungi belong to *Aspergillus* spp., *Fusarium* sp., *Rhizopus* sp., *Penicillium* sp., and *Trichoderma* sp., (Table 1). Three species of *Aspergillus* viz., *A. niger, A. flavus,* and *A. terreus* were found associated with chilies. More than 50% of red chili showed the presence of *A. niger, Penicillium* spp*.,* and *A. flavus* with the frequency of 66.6%, 57.1% and 57.1% respectively. Above 15 % samples showed the presence of *Trichoderma* and *Rhizopus* with occurrences of 28.57% and 19.04%. The *Aspergillus terreus, Fusarium solani* and *Mucor* sp., were isolated from less than 5% of samples (Fig. 1). Our finding is in agreement with the work of Wikandari *et al.*, (2020) mentioning the presence of five genera of fungi including *Penicillium, Eurotium, Fusarium, Mucor,* and *Rhizopus* from the chili of the traditional market. Costa *et al.*, (2019) isolated the least frequency of *Mucor*, *Rhizopus*, *Harzia* and *Cladosporium* genera in different products of *Capsicum* (red chili). Mandeel (2005) evaluated 17 different fungal species and concluded that red pepper (*Capsicum* spp.) was heavily infected by *A. flavus* (96 strains) and *A. niger* (62 strains). Many researchers like Bokhari (2007), Singh *et al*., (2008), and Al-Hindi *et al.*, (2018) reported that *Aspergillus* is the most widespread and dominant genus causing maximum infection contrasted to other genera.

In this study, the highest colony-forming unit (cfu) of *A. niger* (70.0×10^2cfu g^{-1}) were found in longi variety from Kunri followed by *Penicillium* sp. (54.5×10^2 $cfug^{-1}$) from Sakro and *A. flavus* (46.5×10^2 cfu g^{-1}) from Kunri (Table 1). Akhund *et al.*, (2017) reported the 93% occurrence and 2.14×10^4cfu of *A. flavus* in red chili of Kunri. Our results indicated that *A. niger was* the most predominant species and found in all samples of red chili. Similar findings were also reported by Al-Hindi *et al.*, (2018) that the most frequently found genera in red chili are *Aspergillus, Penicillium, Fusarium* and *Rhizopus* with two species of *Aspergillus* viz., *A. flavus* and *A. ochraceus.*

Pods are usually sold in unpacked form, while ground (powder) chili is sold in both unpacked and packed form. The comparative study of both forms showed the greater presence of toxigenic fungal isolates in unground relatively to powdered red chili samples. In this study, longi variety (unground) sample of red chili was heavily contaminated with *A. niger* (70.0×10^2 $cfug^{-1}$) and *A. flavus* (46.5×10^2cfu g^{-1}) (Table 2). Wikandari (2020) reported that total fungal infestation ranged from 0.1 to $40.8 \times 10^4$$cfug^{-1}$ in chilies obtained from both the traditional and the modern markets, where dominant species were found *A. parasiticus* and *A. flavus*.

Table 1. Incidence of fungal species associated with chili peppers collected from different localities of Pakistan.

Location	Sample*	Local names	*Aspergillus niger*	*Aspergillus flavus*	*Trichoderma* sp.	*Fusarium solani*	*Penicillium* sp.	*Rhizopus* sp.
			CFUg^{-1}					
Kunri	CL11	Longi	_	_	_	_	1.0×10^2	_
	CL16	Longi	70.0×10^2	_	_	_		_
	CL17	Longi	4.5×10^2	2.0×10^2	_	_	1.0×10^2	0.5×10^2
	CL21	Longi	7.0×10^2	46.5×10^2	_	_	0.5×10^2	_
Kunri Black	CL13	Longi	_	_	_	0.5×10^2	_	_
	CL19	Longi	11.5×10^2	_	_	_	3.0×10^2	_
Moro	CL12	Whole long	22.0×10^2	0.5×10^2	_	_	-	1.0×10^2
Sakro	CL14	Whole long	7.0×10^2	1.5×10^2	2.0×10^2	_	54.5×10^2	_
Sargodha	CL15	Whole long	_	_	6.5×10^2	_	1.5×10^2	_
	CL20	Whole long	0.5×10^2	_	_	_	_	_
Quetta	CL18	Whole long	15.0×10^2	31.0×10^2	_	_	_	0.5×10^2

*Kunri chili (longi) CL 11, CL 16, CL17, CL 21; Kunri chili black CL 13, CL 19; Moro chili CL 12; Sakro chili CL 14; Sargodha CL15, CL 20; Quetta CL 18

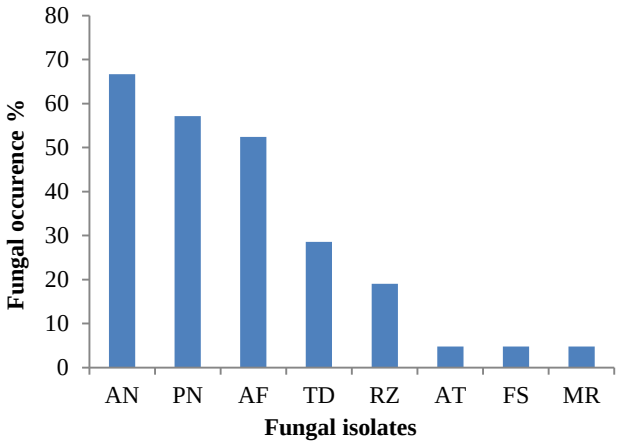

Fig. 1. Percent incidence of fungi present in different samples of chili (*Capsicum*).
*Aspergillus niger (*AN), *Penicillium* (PN), *A. flavus* (AF), *Trichoderma* (TD), *Rhizopus* (RZ), *A. terreus* (AT*), Fusarium solani* (FS), *Mucor* (MR)).

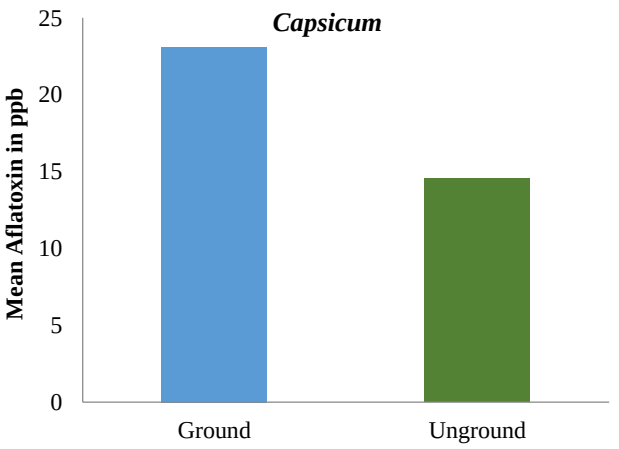

Fig. 2. Mean aflatoxin (µg kg^{-1}) in ground and unground red chili (*Capsicum*).

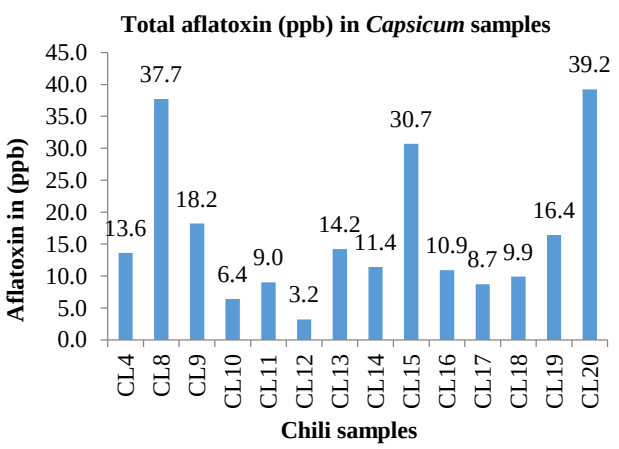

Fig. 3.Total aflatoxin (ppb) in different samples of red chili (*Capsicum*).
Ground (powder) Chili: Commercial powder 2, Commercial powder 1, Commercial powder 3 CL 9; Unground pod chili: Desi chili CL 10, Kunri CL 11 , Moro chili CL 12, Kunri black 1 CL 13, Sangri (Sakro) CL 14, Sargodha chili CL 15, CL16 = Kunri Chili (A), CL17 = Kunri chili (M), CL18 = Gola Quetta, CL19 = Kunri black 2, CL20 = Sargodha chili (Mix quality).

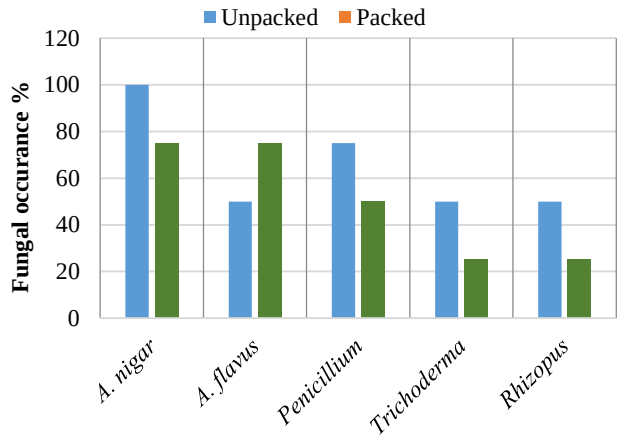

Fig. 4. Occurrence of fungi in different chili samples packed and unpacked.

Aspergillus flavus and *A. parasiticus* are recognized for the production of aflatoxins including aflatoxin B_1 which is highly toxic and carcinogenic mycotoxins (Su, 2019). Plants susceptible to different filamentous fungi, allow them to produce different type of mycotoxins, including aflatoxins B_1, B_2, G_1, G_2, M_1, M_2, trichothecenes, zearalenone, fumonisin, ochratoxin A, T-2 Toxins and Patulin (Anon. 2003; Mandeel, 2005). Aflatoxins in chili are a big threat to human health and a constraint for its export (Paterson, 2007). In this study, *A. flavus* was found 56 % in the ground and 50% in the unground red chili (Table 2). On the contrary, the mean aflatoxin level measured in ground chili was 23.1 µg kg^{-1} and 14.54 µg kg^{-1} in the unground, which is above the European Union limits 10 µg kg^{-1} (Anon., 2010; Iqbal *et al.*, 2010) (Fig. 2). Iqbal *et al.*, (2011) reported a higher concentration of aflatoxin in ground chili as compared to unground (whole) chili. It has been reported that processed *Capsicum* products like (paprika, powdered and crushed pepper) are at risk of aflatoxin contamination than fresh fruit (Iqbal *et al.*, 2011; Khan *et al.*, 2014; Gherbawy*et al.*, 2015). Similarly, Costa *et al.*, (2019) analyzed the presence of mycotoxins in *Capsicum* pepper and found the highest concentration of AFB_1 in red chili powder (35.77 µg/kg) then the red chili flakes (11.45 µgkg^{-1}). In this study, total aflatoxin (AFs) contamination was higher in unground chilies from Sargodha and lowest from Moro followed by in ascending order from Kunri, Quetta, and Sakro (Fig. 3).

In this study, the amount of total aflatoxin ranged from (3.2-39.2 *µgkg^{-1}*) in different samples of red chili *(Capsicum)* as presented in Figure 3. The highest aflatoxin level was detected in chili from Sargodha (Mix powder) (39.2 µgkg^{-1}) followed by in a packed (commercial powder) (37.7 µgkg^{-1}). Paterson, (2007), reported that red chili production is seriously hampered in Pakistan by higher contamination of aflatoxin (Fig. 4) showed the relative study of fungal contamination in local and packed red chilies. The local red chilies were highly susceptible to *A. niger, Penicillium, Trichoderma* and *Rhizopus* sp., where *A. flavus* was found higher in packed red chili. Unpacked red chili available in the local market [week-days bazaar, different kiryana (grocery) shops and supermarkets] showed a higher frequency of fungal growth in local chilies as compared to the packed chili. Detailed investigation is needed to compare the aflatoxin contamination in chili from the local market and packed chili sale by the small stores to superstore.

Table 2. Comparative analysis of fungal species isolated from ground (powder) and unground (whole) chili's samples from different areas of Karachi.

Samples	Sample*	Texture/ shape	*A. niger*	*A. flavus*	*A. terreus*	*Trichoderma* sp.	*F. solani*	*Penicillium* sp.	*Mucor* sp.	*Rhizopus* sp.
			CFUg^{-1}							
Chili powder (ground)	CL1	Packed	3.0×10^2	0.5×10^2	-	-	-	0.5×10^2	-	-
	CL2	Loose	22.0×10^2	0.5×10^2	-	-	-	-	-	1.0×10^2
	CL3	Loose	0.5×10^2	-	-	0.5×10^2	-	1.5×10^2	-	-
	CL4	Packed	$20. \times 10^2$	-	-	0.5×10^2	-	-	-	-
	CL5	Packed	-	-	-	-	-	0.5×10^2	-	-
	CL6	Loose	1.5×10^2	3.0×10^2	-	17.0×10^2	-	0.5×10^2	-	-
	CL7	Loose	0.5×10^2		-	-	-	0.5×10^2	-	0.5×10^2
	CL8	Packed	15.0×10^2	31.0×10^2	-	-	-	-	-	0.5×10^2
	CL9	Packed	-	0.5×10^2	-	-	-	-	-	-
Chili pod (unground)	CL10	long	-	0.5×10^2	-	-	-	-	-	-
	CL11	long	-	-	-	-	-	0.10×10^2	-	-
	CL12	Round	-	6.5×10^2	1.5×10^2	-	-	-	-	-
	CL13	Round	-	-	-	-	0.5×10^2	-	-	-
	CL14	Long	7.0×10^2	1.5×10^2	-	2.0×10^2	-	54.5×10^2	-	-
	CL15	Long	-	-	-	6.5×10^2	-	1.5×10^2	-	-
	CL16	Round	70.0×10^2	46.5×10^2	-	-	-		-	-
	CL17	Round	4.5×10^2	2.0×10^2	-	-	-	1.0×10^2	-	0.5×10^2
	CL18	Round	0.5×10^2	1.0×10^2	-	9.0×10^2	-	1.5×10^2	0.5×10^2	-
	CL19	Round	11.5×10^2	-	-	-	-	3.0×10^2	-	-
	CL20	Long	0.5×10^2	-	-	-	-	-	-	-
	CL21	Round	7.0×10^2	-	-	-	-	0.5×10^2	-	-

*Samples collected from: Large superstore CL 1, Small karyana store CL 2, Large karyana store CL 3, Commercial powder (2) CL 4 , Small super store CL 5, Weekdays bazar chili (1) CL 6 , Weekdays bazar chili (2) CL 7, Commercial powder (1) CL 8 , Commercial powder (3) CL 9; Chili unground pod round (CL-12, CL 13, CL 16, CL 17, CL 18, CL 19 and CL 21) Chili unground pod long (CL 10, CL 11, CL 14, CL 15, CL 20)

Conclusion

It is interesting to note that contamination of aflatoxin in most of the chili samples (37.5%) was found below 10ppb, which is under E.U legislative allowable limits. Whereas, United States Department of Agriculture (USDA) allows up to 20 µgkg^{-1} (ppb) of total aflatoxin (AF) levels. Pakistan's chili farmers are availing the opportunity to enter the lucrative export market and in order to increase their market share, they need to continue producing high-quality products. Chili traders in Pakistan should be careful about the quality selection of chili pod which is free from fungal contamination and aflatoxin. There is great potential to increase the export to the European market if the required standards are met. One of the most important quality attributes for chili is the aflatoxin content, which should be below the acceptable limit.

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