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Detection of Mycoflora and Aflatoxin B_1 in the Seeds of Inodorous Melons (*Cucumis melo* L.)

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Abstract

Twenty-two seed samples of inodorous melons, collected from the areas of Peshawar, Swabi, Tordher, Fatu-chuk, Mardan, Karachi, Islamabad, Ghotki, and Mandibahauddin, yielded 75 species of 36 fungal genera, isolated through ISTA (International Seed Testing Association) techniques. The agar plate method was chosen as being best for the qualitative and quantitative isolation of fungi, followed by the standard blotter method. The agar plate method yielded 64 species of 29 genera, while the blotter method yielded 24 species belonging to 14 genera. The deep-freezing method yielded only 2 species belonging to 2 genera. *Aspergillus niger*, followed by *A. flavus*, *Chaetomium globosum*, and *Rhizopus stolonifer* were the most dominant fungi in all 3 methods used. Forty species belonging to 25 genera had not been previously reported from Pakistan. Seven seed samples, which were highly infected with fungi, were grown in test tube slants, included samples from Tordher (1), Ghotki (1), Mandibahauddin (1), Karachi (2), Islamabad (1), and Fatu-chuk (1). *Aspergillus flavus* was the most dominant fungi, causing pre-emergence rot of seedlings. *Fusarium oxysporum* caused 3.6 % of seedling deaths after 10 - 12 days of incubation. Seed samples from Islamabad, Mandibahauddin, and Swabi were highly infected with *A. flavus*. The level of aflatoxin B_1 estimated through CD-ELISA for the 3 samples was 32.64 ppb (Swabi), 11.48 ppb (Islamabad), and 7.30 ppb (Mandibahauddin), respectively, of which the seed sample from Swabi contained the highest level of aflatoxins. Surface sterilization of seeds with 1 % Calcium hypochlorite ($Ca(OCl)_2$) greatly reduced the incidence of both saprophytic and superficial pathogenic fungi.

Keywords: Aflatoxins, ELISA, inodorous melons, ISTA techniques, seed-borne mycoflora

Introduction

Cucumis melo L. is a member of the family cucurbitaceae, commonly known as melon. Melon is a diverse group of fleshy fruits, which includes cantaloupes (muskmelons), inodorous melons or winter melons (Honeydew, Juan Canary melons, and mixed varieties of melons, like Casaba, Crenshaw, Persian, Santa Claus etc.), and water melons [1-3]. Commonly, melons of all types are called kharbooza in South-East Asia. Depending upon the variety, melons can have musky odor, or can be without any odor. The skin (rind) of melon can be hard, reticulate, netted, lined, or plain smooth [4]. Melon is native to Africa [5] and southwest Asia [6]. Nowadays, melons are cultivated in many parts of the world. China (25 billion pounds), Turkey (3.5 billion pounds), Egypt (2.9 billion pounds), and the U.S. (2.2 billion pounds) are the major melon producing countries of the world [7]. During 2010 - 2011, Pakistan produced around 23,898 tonnes of melons cultivated on 15,918 hectares of land [8]. Melon grows well in hot, sunny, and dry weather, and can be cultivated on well-drained loam to clay-loam soils [9]. Melon is a nutritious fruit, and provides a wide range of nutrients, minerals, vitamins, anti-oxidants, etc. Melon is a good source of carbohydrates, sugars, starch, fats, dietary fiber, proteins, Vitamin A, Vitamin B_1 (thiamine), B_2

(Riboflavin), B_3 (niacin), B_5 (Pantothenic acid), B_6, B_9 (Folate), B_{12} (cobalamin), vitamin C, Vitamin E, vitamin K, calcium, iron, magnesium, manganese, potassium, zinc, copper, sodium, phosphorous, fluoride, and selenium. Melon seeds are edible and contain oil, and are known as chaar-maghaz. Melon seeds contain fats, carbohydrates, calcium, iron, protein, Vitamin B_1, B_2, B_3, B_5, B_6, B_9, magnesium, phosphorous, potassium, sodium, zinc, copper, and manganese. The seeds can help against high cholesterol levels, nervous disorders, cardiovascular disorders, and help strengthen the immune system [10-12]. The seeds are used in homeopathic [13] and ayurvedic medicines [14]. Shelled seeds are used in making cakes, biscuits, and curries, and as gravy thickener. Seeds are added to halwas and sweets in the sub-continent. Imitation jewelry is made from melon seeds [15], cosmetics, and herbal oils [16,17]. A study of literature shows that rot of fungi (both pathogenic and saprophytic) is associated with various melon cultivars. These fungi are responsible for causing root rot, fruit rot, vine decline, post-harvest rot, etc. These fungi are usually present inside seeds as dormant mycelium. Seeds are important disease reservoirs, while some of these fungi can cause serious damage to future crops by reducing the quality and quantity of seeds [18,19]. Aegerter *et al.* [20] reported root rot and vine decline in melons in California, due to *Acremonium cucurbitacearum*, *Rhizopycnis vagum*, *Monosporascus cannonballus*, *Fusarium solani*, *Macrophomina phaseolina*, *Pythium* spp., *Rhizoctonia solani*, and *Verticillium dahlia*. Chiejina [18] isolated 11 fungal species, viz; *Rhizopus stolonifer*, *Cunninghamella* sp., *Aspergillus niger*, *Penicillium* sp., *Mucor* sp., *Trichoderma* sp., *Curvularia* sp., *Fusarium oxysporum*, *Aspergillus flavus*, *Syncephalastrum* sp., and *Aspergillus* sp., from 3 cultivars of melons seeds collected from Nigeria. *Alternaria alternata* and *A. cucumerina* caused black mould disease in melons [21]. Jimenez *et al*. [22] reported the death of melon vines in Spain due to *A. cucurbitacearum*, *M. cannonballus*, *M. phaseolina*, and *R. solani*; however, *R. vagum* and *Plectosporium tabacinum* were also responsible for the death of melon plants. Ekundayo and Idzi [23] isolated 13 fungi from moldy-shelled melon seeds collected from Nigeria. The isolated fungi included the species of *Mucor*, *Rhizopus, Aspergillus*, *M. phaseolina*, *Penicillium*, *Alternaria*, *Fusarium*, *Botrytis*, *Torula*, and *Geotrichum.* When these fungi were artificially re-inoculated to healthy shelled melon seeds, free fatty acids increased after 7 - 14 days*. F. solani*, followed by *Aspergillus* and *Penicillium*, produces the highest values of free fatty acid contents. Bankole *et al.* [24] reported that, during prolonged storage of melon seeds in polyethylene as well as jute bags, field fungi like *Alternaria, Botryodiplodia theobromae*, *Cladosporium*, *Fusarium*, and *M. phaseolina* gradually decreased, while storage fungi like *Aspergillus*, *Penicillium*, and *Rhizopus* increased. Bankole *et al.* [25] isolated species of *Aspergillus*, *Botryodiplodia*, *Cladosporium*, and *Rhizopus* from the shelled melon seeds (*Citrullus colocynthis* L.). *A. flavus* caused high infection; also, the seeds had aflatoxin B_1. Bankole [26] also reported decrease in seed germination and moisture content during prolonged storage of seeds, along with increase in mould content. Fatima *et al.* [27] isolated *Cladosporium cladosporioides*, *Fusarium solani*, and *Geotrichum candidum* as being responsible for the post-harvest rot of melon fruits collected from Karachi, Pakistan. Ahmad *et al*. [28] reported *Fusarium nivale* and *Myrothecium roridum* as seed-borne fungi of long melon (*Cucumis melo*)*.* More than 300 fungal species produce mycotoxins [29]. Mycotoxins are low weight diverse chemical compounds produced as secondary metabolites by numerous fungi in a variety of food commodities, as well as in growing crops and during storage, which are equally harmful to animals, plants, and humans [30]. The purpose of the current work was to identify the seed-borne mycoflora and the aflatoxins produced in plain yellow skinned, inodorous melons cultivated in Pakistan.

Materials and methods

Seed-borne mycoflora was detected through ISTA (International Seed Testing Association) techniques [31] Using the standard blotter method, the Agar plate method, and the deep-freezing method, as suggested by ISTA, 400 seeds of each sample were tested.

Collection of seeds

Twenty two seed samples of melon were collected from various areas of Pakistan, *viz;* Peshawar (1), Swabi (1), Tordher (1), Fatu-chuk (1), Mardan (2), Karachi (13), Islamabad (1), Ghotki (1), and Mandibahauddin (1). All the collected seed samples were stored at room temperature (15 - 35 °C) in well labeled air-tight glass jars for future use.

Standard blotter method

Non-surface disinfected and seeds surface disinfected with 1 % $Ca(OCl)_2$ for 2 min, placed on 3 layers of moistened blotter paper with 10 seeds per Petri dish. The dishes were incubated for 5 - 7 days at 28 ± 2 °C under 12 h, an alternating cycle of artificial day light (ADL) and darkness [31].

Agar plate method

Non-surface sterilized and seeds surface disinfected with 1 % $Ca(OCl)_2$ for 2 min, placed aseptically on sterile Potato dextrose agar (PDA), with 10 seeds per Petri dish. The dishes were incubated for 5-7 days at 28±2 °C under 12 h, an alternating cycle of artificial day light (ADL) and darkness [31].

Deep-freezing method

Seeds non-surface sterilized and after sterilization with 1 % $Ca(OCl)_2$ for 2 min, placed aseptically on three layers of moistened blotter paper. Ten seeds per Petri dish were incubated for 24 h, each at 28±2 °C and –2 °C followed by 5 days incubation at 28±2 °C under 12 h, an alternating cycle of artificial day light (ADL) and darkness [31].

Seedling symptoms test

For the pre- and post-emergence rot of seeds and seedlings due to fungal infection, a seedlings symptoms test was carried out for 7 seed samples selected from each crop. 100 seeds were randomly selected from each sample. Fifty seeds were washed with sterilized distilled water, and the other 50 seeds were surface sterilized with 1 % $Ca(OCl)_2$ for 5 min. Seeds were placed aseptically in sterilized test tube slants containing 2 % plain water agar, at a rate of one seed per test tube. The mouths of the test tubes were covered with loose cotton plugs. The test tubes were incubated for 14 days at an ambient temperature (28±2°C) under 24 h alternating cycles of artificial day light (ADL) and darkness. Test tubes were unplugged when seedlings reached the mouths of the test tubes [32].

Estimation of Aflatoxin B_1

For randomly selected seed samples, quantitative analysis of aflatoxin B_1 was done through CD-ELISA, by using commercially available immunoassay kit Veratox. Using Log/logit software, the concentration of Aflatoxin B1 was calculated [35].

Identification of fungi

Mycoflora observed on seeds were identified after reference to Barnett and Hunter [34], Booth [36], Domsch *et al*. [37], Ellis [38], Gilman [39], Hanlin [40], Mycobank [42], Nelson *et al*. [41], and Raper *et al*. [43].

Analysis of data

For the calculations of ANOVA, procedures suggested by Gomez & Gomez [44] and Sokal and Rohlf [45] were followed.

Results

At least 75 species belonging to 36 genera, viz; *Absidia corymbifera* (Cohn) Sacc. & Trotter, *A. cylindrospora* Hagem, *A. spinosa* Lendner, *Absidia* species., Van Tieghem, *Acremonium rutilum* W. Gams, *Acremonium* Link ex Fr., *Aspergillus alutaceous* Berk. & Curt., *A. erythrocephalus* Berk. & Curt., *Aspergillus flavus* Link ex Gray., *A. fumigatus* Fres., *A. niger* Van Tieghem, *A. ochraceous* Wilhelm, *A. oryzae* (Ahlburg) Cohn., *A. parasiticus* Speare, *A. terreus* Thom, *A. ustus* (Bain) Thom & Church, *A.versicolor* (Vuill.) Tiraboschi, *A. wentii* Wehmer, *Botrytis cinerea* Pers. ex Nocca & Balb., *Botriotrichum piluliferum* Sacc. & March, *Chaetomium bostrychodes* Zopf., *C. crispatum* (Fuckel) Fuckel, *C. elatum* Kunze ex Steud., *C. funicola* Cooke, *C. globosum* Kunze ex steud., *C. indicum* Corda, *C. murorum* Corda, *C. spirale* Zopf, *Chaetomium* species Kunze ex Fr., *Cladosporium cladosporioides* (Fres.) de Vries., *C. cucumerinum* Ellis & Arth., *C. macrocarpum* Preuss, *C. oxysporum* Berk & Curt, *C. spaerospermum* Penz., *Cladosporium* sp. Link ex Fries; Link, *Curvularia pallescens* Boedijn, *C. penniseti* (Mitra) Boedijn, *Drechslera cynodontis* (marignoni) Subram. & Jain., *Drechslera* sp. Ito., *Dicyma ampullifera* Boulanger, *Emericella nidulans* (Eidam) Vuill., *Emericella* sp., Berk & Br., *Eurotium* spp., Link ex Gray, *Fulvia fulva* (Cooke) Ciferri, *Fusarium oxysporum* Schlecht. emend. Sny. & Hans., *F.solani* (Mart.) Sacc., *Fusarium* species Link ex Fr., *Gilmaniella humicola* Barron, *Lophotrichus ampullus* R.K. Benjamin, *Macrophomina phaseolina* (Tassi) Goid, *Microascus cirosus* Zukal, *Melanospora* sp. Corda, *Memnoniella echinata* (Riv.) Galloway, *Monilia* sp. Pers. ex Fr., *Monoascus* sp. Van Tiegh, *Myrothecium cinctum* (Corda) Sacc., *Papulaspora irregularis* Hotson, *Penicillium nigricans* Bain ex Thom, *Penicillium* species Link ex Fr., *Phoma glomerata* (corda) Wollenw & Hochapfel., *P.pomorum* Thüm, *Phoma* sp. Sacc., *Pithomyces* species Berk. & Br., *Pseudogymnoascus roseus* Raillo, *Rhizopus oryzae* Went & Prinsen Geerligs, *R. stolonifer* (Ehrenb. Ex Link) Lind, *Sagnomella diversispora* (Van Beyma) W. Gams, *Scytillidium lignicola* Pesante, *Thelavia* species Zopf., *Trichoderma hamatum* (Bonord.) Bain, *T. harzianum* Rifai, *T.polysporum* (Link ex Pers.) Rifai, *Trichosporiella cerebriformis* (de Vries. & kleine. Natrop) W. Gams, *Ulocladium consortiale* (Thüm.) Simmons, and *Wallemia sebi* (Fr.) V. Arx, were isolated from the seed samples collected from various areas of Pakistan by using ISTA techniques (**Table 1**). The agar plate method, followed by the standard blotter method, was best for the isolation of fungi, both qualitatively as well as quantitatively ($P < 0.001$). The agar plate method yielded 64 species belonging to 29 genera, while the blotter method yielded 24 species belonging to 14 genera. The deep-freezing method yielded only 2 species belonging to 2 genera. *A. niger* ($P < 0.001$), followed by *A. flavus* ($P < 0.001$), was the most dominant fungus (**Table 2**). *Chaetomium globosum* and *Rhizopus stolonifer* were equally responsible for seed infection. Surface sterilization of seeds with 1 % $Ca(OCl)_2$ reduced the infection percentage of *Aspergillus, Chaetomium, Rhizopus*, and *Trichoderma* species; however, overall, quantitatively greater numbers of fungi were isolated after surface sterilization. Species of *Fusarium* and *Phoma* were isolated through the agar plate method, while *Macrophomina phaseolina* was isolated through the blotter method. Seeds samples from Peshawar, Tordher, Mandibahauddin, Islamabad, Swabi, and 4 samples from Karachi, were highly infected with fungi. Keeping in view all of the previously reported work, 40 fungal species of 25 genera had not been previously reported in Pakistan.

Seed samples heavily infected with *Aspergillus* species, besides other fungi, were grown in test tubes to check the fungal growth. Samples from Tordher (1), Ghotki (1), Mandibahauddin (1), Karachi (1), Islamabad (1), Swabi (1), and Fatu-chuk (1) were grown aseptically on 2 % plain water agar. Species of *Acremonium*, *Aspergillus*, *Chaetomium*, *Drechslera*, *Monodyctis*, *Myrothecium*, *Penicillium*, *Rhizopus*, *Scopulariopsis*, and *Trichoderma* caused pre-emergence death of seeds. *A. flavus* caused the highest infection (12.73 %), followed by *Penicillium* (5.45 %). *A. fumigatus* caused 5.45 % infection in surface sterilized seeds. Ascomycetes like *Emericella rugulosa*, *Microascus* sp., *Neocosmospora vasinfecta* and *Thielavia terricola* caused pre-emergence death of seeds, without producing any visible symptoms. *F. oxysporum* caused 3.6 % of the seedling deaths after 10 - 12 days of incubation. *M. phaseolina* produced minor infections (1.81 %) in non-surface sterilized seeds; however, sclerotia were visible to naked eye. *Chaetomium* species produced infection in seedlings at a minor level. Surface sterilization has greatly reduced the incidence of fungi, both pathogenic and saprophytic. After 14 days of incubation, 34.55 % of seedlings remained healthy in non-surface sterilized seeds, while 38.18 % of seedlings remained healthy after surface sterilization (**Table 3**). Out of 7 samples tested, seeds from Islamabad, Mandibahauddin, and Swabi had high infection due to *A. flavus*. These 3 samples were tested for aflatoxin B_1 through an Competitive Direct Enzyme Linked Immunosorbent Assay (CD-ELISA) using a commercially available

Veratox kit. Seeds from Swabi had the highest levels of aflatoxin B_1 (32.44 ppb), while seed samples from Islamabad and Mandibahauddin had estimated values of 11.48 and 7.30 ppb, respectively. The estimated values of aflatoxin B_1 in seed samples from Swabi were above the permissible limit, as recommended by United States Food and Drug Administration (**Table 4**).

Table 1 Detection of seed-borne fungi in inodorous melons (*Cucumis melo* L.) using ISTA technique.

Name of fungi	Standard blotter method				Agar plate method				Deep-freezing method			
	NSt		SSt		NSt		SSt		NSt		SSt	
	NSI	I% ± S.D	NSI	I% ± S.D	NSI	I% ± S.D	NSI	I% ± S.D	NSI	I% ± S.D	NSI	I% ± S.D
*Absidia corymbifera**	-	-	-	-	1	0.11±0.00	1	0.69±0.00	-	-	-	-
*A.cylindrospora**	-	-	-	-	1	0.04±0.00	-	-	-	-	-	-
*A.spinosa**	-	-	-	-	-	-	1	0.73±0.00	-	-	-	-
Absidia sp.*	-	-	-	-	2	0.84±12.02	4	0.95±9.04	-	-	-	-
Acremonium rutilum	-	-	-	-	1	0.04±0.00	-	-	-	-	-	-
Acremonium sp.	-	-	-	-	-	-	1	0.15±0.00	-	-	-	-
Aspergillus alutaceus	1	0.04±0.00	1	0.04±0.00	1	0.04±0.00	1	0.07±0.00	-	-	-	-
A.erythrocephalous	2	0.07±0.00	1	0.04±0.00	-	-	-	-	-	-	-	-
A.flavus	12	4.75±7.48	12	4.42±23.1	19	21.5±21.10	19	15.7±20.00	1	0.10±0.00	1	0.04±0.00
A.fumigatus	-	-	-	-	4	0.77±3.86	4	0.62±3.30	-	-	-	-
A.niger	6	0.86±4.90	4	0.25±1.50	18	30.3±29.00	17	20.0±43.60	-	-	-	-
A.ochraceous	-	-	-	-	2	0.07±0.00	-	-	-	-	-	-
A.oryzae	1	0.43±0.00	1	0.97±0.00	-	-	-	-	-	-	-	-
A.parasiticus	-	-	-	-	-	-	3	0.18±0.6	-	-	-	-
A.terreus	-	-	-	-	5	0.95±8.3	6	1.24±5.40	-	-	-	-
A.ustus	-	-	1	0.07±0.00	1	0.04±0.00	-	-	-	-	-	-
A.versicolor	-	-	-	-	1	0.04±0.00	1	0.11±0.00	-	-	-	-
A.wentii	1	0.04±0.00	-	-	7	0.77±3.40	2	0.29±1.4	-	-	-	-
Botrytis cinerea	-	-	-	-	1	0.04±0.00	-	-	-	-	-	-
*Botryotrichum piluliferum**	-	-	-	-	-	-	1	0.65±5.70	-	-	-	-
*Chaetomium bostrychodes**	-	-	1	0.04±0.00	-	-	-	-	-	-	-	-
*C.crispatum**	-	-	-	-	2	0.26±3.50	-	-	-	-	-	-
*C.elatum**	1	0.36±0.00	2	0.61±6.02	-	-	1	0.62±0.00	-	-	-	-
*C.funicola**	-	-	-	-	-	-	1	0.11±0.00	-	-	-	-
*C.globosum**	2	1.04±19.10	2	0.86±15.60	4	0.55±1.7	1	0.26±0.00	-	-	-	-
*C.indicum**	-	-	-	-	1	0.11±0.00	1	0.04±0.00	-	-	-	-
*C.murorum**	-	-	-	-	1	0.04±0.00	-	-	-	-	-	-
*C.spirale**	1	0.29±0.00	-	-	-	-	-	-	-	-	-	-
Chaetomium spp.*	3	0.25±1.53	-	-	1	0.04±0.00	1	0.99±13.4	-	-	-	-
Cladosporium cladosporioides	-	-	-	-	-	-	1	0.29±4.20	-	-	-	-
C.cucumerinum	-	-	-	-	-	-	1	0.07±0.00	-	-	-	-
C.macrocarpum	-	-	-	-	-	-	1	0.04±0.00	-	-	-	-
C.oxysporum	-	-	1	0.04±0.00	-	-	1	0.07±0.00	-	-	-	-
C.sphaerospermum	-	-	-	-	-	-	1	0.07±0.00	-	-	-	-
Cladosporium sp.	-	-	1	0.04±0.00	1	0.04±0.00	1	0.08±0.00	-	-	-	-
Curvularia pallescens	-	-	-	-	-	-	1	0.109±0.00	-	-	-	-
C.pennisiti	-	-	-	-	-	-	1	0.04±0.00	-	-	-	-
*Dicyma ampullifera**	-	-	-	-	-	-	1	0.04±0.00	-	-	-	-
*Drechslera cynodontis**	-	-	-	-	-	-	1	0.18±0.00	-	-	-	-
Drechslera sp.*	-	-	-	-	-	-	1	0.11±0.00	-	-	-	-
*Emericella nidulans**	-	-	-	-	1	0.04±0.00	2	0.14±1.40	-	-	-	-
Emericella sp.*	-	-	-	-	-	-	2	0.14±1.40	-	-	-	-
Eurotium sp.*	-	-	-	-	-	-	1	0.26±0.00	-	-	-	-
*Fulvia fulva**	-	-	-	-	1	0.04±0.00	-	-	-	-	-	-
Fusarium oxysporum	-	-	-	-	-	-	1	0.26±0.00	-	-	-	-
F.solani	-	-	-	-	-	-	1	0.04±0.00	-	-	-	-
Fusarium sp.	-	-	-	-	-	-	1	0.07±0.00	-	-	-	-
*Gilmaniella humicola**	-	-	1	0.04±0.00	-	-	-	-	-	-	-	-
*Lophotrichus ampullus**	-	-	1	0.18±0.00	-	-	2	0.19±2.12	-	-	-	-
Macrophomina phaseolina	-	-	1	0.04±0.00	-	-	-	-	-	-	-	-

Name of fungi	Standard blotter method				Agar plate method				Deep-freezing method			
	NSt		SSt		NSt		SSt		NSt		SSt	
	NSI	I% ± S.D	NSI	I% ± S.D	NSI	I% ± S.D	NSI	I% ± S.D	NSI	I% ± S.D	NSI	I% ± S.D
Melanospora sp. *	2	0.07±0.00	-	-	-	-	-	-	-	-	-	-
*Memnoniella echinata**	-	-	-	-	-	-	1	0.04±0.00	-	-	-	-
*Microascus cirosus**	-	-	-	-	-	-	1	0.04±0.00	-	-	-	-
Monilia sp.*	-	-	-	-	-	-	1	0.73±0.00	-	-	-	-
Monoascus sp.*	-	-	1	0.04±0.00	-	-	-	-	-	-	-	-
Myrothecium cinctums	-	-	-	-	1	0.11±0.00	2	0.98±14.90	-	-	-	-
*Papulaspora irregularis**	1	0.07±0.00	-	-	-	-	-	-	-	-	-	-
Penicillium nigricans	-	-	-	-	1	0.07±0.00	-	-	-	-	-	-
Penicillium sp.	-	-	-	-	1	0.22±0.00	2	0.37±1.40	-	-	-	-
*Phoma glomerata**	-	-	-	-	1	0.04±0.00	-	-	-	-	-	-
*P.pomorum**	-	-	-	-	-	-	1	0.07±0.00	-	-	-	
Phoma spp.*	-	-	-	-	2	0.95±0.00	1	0.04±0.00	-	-	-	-
Pithomyces sp.*	-	-	-	-	-	-	1	0.08±0.00	-	-	-	-
*Pseudogymnoascus reseus**	-	-	-	-	1	0.07±0.00	-	-	-	-	-	-
Rhizopus oryzae	2	0.18±2.08	1	0.07±0.00	3	0.77±3.00	3	0.37±1.50	-	-	-	-
R.stolonifer	2	0.54±7.78	1	0.43±0.00	9	1.61±3.40	3	0.73±8.90	1	0.04±0.00	-	-
*Sagnomella diversispora**	-	-	1	0.90±0.00	-	-	-	-	-	-	-	-
*Scytillidium lignicola**	-	-	-	-	-	-	1	0.04±0.00	-	-	-	-
Thielavia sp.*	-	-	-	-	1	0.04±0.00	-	-	-	-	-	-
Trichoderma hamatum	-	-	-	-	2	0.32±3.50	1	0.80±0.00	-	-	-	-
T.harzianum	1	0.07±0.00	-	-	2	0.64±4.90	-	-	-	-	-	-
T.polysporum	-	-	-	-	1	0.04±0.00	-	-	-	-	-	-
*Trichosporiella cerebriformis**	-	-	-	-	-	-	1	0.18±0.00	-	-	-	-
*Ulocladium consortiale**	-	-	-	-	1	0.08±0.00	-	-	-	-	-	-
*Wallemia sebi**	-	-	-	-	1	0.04±0.00	-	-	-	-	-	-
$LSD_{0.05}$: Cond = 0.759; Meth = 0.929												

NSt = Non-surface sterilized seeds; SSt = Surface sterilized seeds: NSI = Number of samples infected; I% = Infection percentage, S.D = Standard Deviation, *= Not previously reported in Pakistan

Table 2 Two way completely randomized ANOVA for fungi isolated by blotter, agar plate, and deep-freezing methods (ISTA techniques) from melon seed samples.

Variable	Source	Sum of squares	df	Mean square	F-value	P-value
Total fungi	Main effects					
	Cond	0.071	1	0.071	0.015	0.901 ns
	Meth	548.904	2	274.452	59.285	0.0000***
	Interaction					
	Cond × Meth	0.428	2	0.214	0.046	0.954 ns
	Error	555.523	120	4.629		
	Total	1104.928	125			
Aspergillus flavus	Main effects					
	Cond	232.071	1	232.071	1.108	0.294 ns
	Meth	13297	2	6648.5	31.751	0.0000***
	Interaction					
	Cond × Meth	379.476	2	189.738	0.906	0.406 ns
	Error	25126.952	120	209.391		
	Total	39035.5	125			
Aspergillus niger	Main effects					
	Cond	709.531	1	709.531	1.583	0.210 ns
	Meth	29564.777	2	14782.388	32.984	0.0000***
	Interaction					
	Cond × Meth	1190.777	2	595.388	1.328	0.268 ns
	Error	53778.120	120	448.157		
	Total	85244.039	125			

*** = Significant at $P < 0.001$ level; ns = Non significant

Table 3 Detection of deep-seated fungi of inodorous melons using seedling symptoms test.

Name of fungi	Pre-emergence rot		Post-emergence rot	
	Infection percentage (%)			
	NSt	SSt	NSt	SSt
Acremonium cerealis	1.81	-	-	-
Aspergillus candidus	1.81	-	-	-
Aspergillus flavus	12.73	3.6	-	-
Aspergillus fumigatus	-	5.45	-	-
Aspergillus niger	1.81	-	-	-
Aspergillus sp.	1.81	-	-	-
Chaetomium bostrychodes	-	1.88	-	-
Chaetomium indicum	-	-	1.81	-
C.jodhpurense	1.81	-	-	-
Chaetomium trilaterale	-	-	1.81	-
Drechslera sp.	1.81	-	-	-
Emericella rugulosa	1.88	-	-	-
Fusarium oxysporum	-	-	3.6	1.81
Fusarium solani	-	-	1.81	-
Macrophomina phaseolina	1.81	-	-	-
Microascus sp.	3.6	-	-	-
Monodyctis sp.	1.81	-	-	-
Myrothecium sp.	1.81	-	-	-
Neocosmospora vasinfecta	-	1.81	-	-
Penicillium sp.	5.45	-	-	-
Rhizopus stolonifer	1.81	-	-	-
Scopulariopsis brevicaulis	1.81	-	-	-
Scopulariopsis brumptii	1.81	-	-	-
Tetracoccosporium paxianum	1.81	-	-	-
Thielavia terricola	3.6	-	-	-
Trichoderma harzianum	-	3.6	-	-
Healthy seedlings (%)	**NSt**		34.55	
	SSt		38.18	

NSt = Non-surface sterilized seeds; SSt = Surface sterilized seeds

Table 4 Estimated values (ppb) of Aflatoxin B_1 in melon seeds through CD-ELISA technique.

Name of seed crop	Locality of seed samples	Aflatoxin B1 ppb ± S.D.
	Permissible level 20 ppb (USFDA)	
Melon	Swabi	32.44 ± 1.112
Melon	Mandibahauddin	7.30 ± 0.576
Melon	Islamabad	11.48 ± 0.561

(ppb = parts per billion; S.D. = Standard Deviation)

Discussion

Seventy five species belonging to 36 fungal genera were isolated from 22 seed samples of inodorous melons collected from selected areas of Pakistan. Species of *Aspergillus*, *Chaetomium*, and *Rhizopus* produced the highest infection, while seed samples from Peshawar, Tordher, Mandibahauddin, and Karachi were highly infected with fungi. Surface sterilization with 1 % $Ca(OCl)_2$ reduced the species of *Aspergillus*, *Chaetomium*, *Rhizopus* and *Trichoderma*; however, a quantitatively greater number of fungi were isolated after surface sterilization. Surface sterilization reduced the growth of fast growing fungi, giving the opportunity of growth to deep-seated slow growing fungi. Rahim *et al.* [46] also reported similar results in pumpkin seeds, Rahim and Dawar [47], as did Rahim *et al.* [48] on lentil seeds, and Niaz and Dawar [49] on maize seeds. Sauer and Burroughs [50] reported that 1 - 5 % concentration of sodium hypochlorite ($Na(OCl)_2$) could readily kill the spores of the *Aspergillus* species. Wilson [51] reported that the use of any concentration of calcium hypochlorite as a surface disinfectant is effective against fungi and bacteria, as well as for enhancement in germination and breaking dormancy. The agar plate method was best for the isolation of fungi, both qualitatively and quantitatively, followed by the blotter method; however, the deep-freezing method yielded the least number of fungi. Melon seeds rotted and decayed when exposed to low temperature, due to bacterial infection. Rahim *et al.* [46] also reported similar results on pumpkin seeds, where pumpkin seeds decayed after freezing. Elwakil and El-Metwally [52] reported the deep-freezing method was best for the isolation of *Aspergillus nidulans*, *A. versicolor*, and *A. carneus*; however, the blotter method was best for the isolation of a greater number of fungi as compared to the deep-freezing method. Lee *et al.* [53] found the blotter method best for the isolation of *Didymella bryoniae* from cucurbit seeds as compared to the agar plate method. Seedling symptoms tests yielded internally deep-seated fungi during 14 days of incubation. Fungi produce mycotoxins, which are secondary metabolites produced by several fungal species. Niaz *et al.* [29] found that 50 seed samples of maize out of 59 were contaminated with mycotoxins from various fungi. If consumed, mycotoxins are harmful to health, and can be fatal at times, to both animals and humans [54]. In addition, mycotoxins reduced the yield, besides the value of the crops [55]. Bankole *et al.* [24] detected aflatoxin B_1 in 32.2 % shelled melon seeds (*Colocynthis citrullus* L.) collected from Nigeria. In Pakistan, low phytosanitary conditions are responsible for high fungal contamination in food and feed stuff. Mushtaq *et al.* [56] detected the presence of aflatoxin B_1 in 125 processed food stuff using the RP-HPLC technique, out of which 21 % of infant food products had high levels of aflatoxins, as compared to European Union permissible levels (0.1 µg/Kg). Rashid *et al.* [57] also reported such similar results, where more than 91 % of poultry feed in Pakistan had aflatoxins, out of which 82 % of samples were above the permissible limits recommended by the United States Food and Drug Administration. Since melon crop is cultivated through seeds, care is required to store the seeds properly. Melon seeds can remain viable for five years when stored in cool, dry, dark conditions [4]. Storage of melons seeds in polyethylene bags is preferable [23,58]. Pakistan is an agricultural country, and the economy greatly depends on agriculture; improving the phytosanitary, conditions can lower the risk to future crop.

Conclusions

Seventy five fungal species belonging to 36 fungal genera were isolated from 22 seed samples collected from various localities of Pakistan. Seed samples found heavily infected with fungi were tested for seedling symptoms tests. Using CD-ELISA technique, seed samples infected with *Aspergillus flavus* were tested for aflatoxin B_1, of which sample from Swabi were found heavily contaminated with aflatoxin B_1.

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