

MYCOFLORA ASSOCIATED WITH RAISINS (*VITIS VINIFERA* L.) COLLECTED ACROSS PAKISTAN

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Abstract

Aim of study was to isolate and identify fungal pathogens associated with raisins (*Vitis vinifera* L.) collected from Punjab, Sindh, Baluchistan, Khyber Pakhtunkhwa provinces of Pakistan. Around 25 fungal species belonging to 15 genera were isolated from the fifteen samples by using ISTA (International Seed Testing Association) techniques. Of these, 19 species belonging to 11 genera were isolated by deep freezing method, agar plate method yielded 16 species belonging to 9 genera and 16 species belonging to 11 genera were isolated by blotter method. Deep freezing method was found best for isolation of fungi followed by standard blotter method. Species of *Aspergillus* and *Penicillium* were the most dominant fungi. Samples of raisins from the areas of Lahore, Islamabad and Karachi, respectively were found to be highly infected with fungi. These samples were treated with Ultra Violet (UV-C) radiations which significantly affected the pathogenic profile, but the conidia of *Aspergillus niger* were appeared to be more persistent that colonized the raisins within the storage time of zero day. However, infection by other storage fungus like *Aspergillus oryzae* (Ahlburg) Cohn. was observed after the storage time of 30 and 60 days.

Key words: Raisins, ISTA technique, Ultra violet radiation, Fungi, Pakistan.

Introduction

Raisins (*Vitis vinifera* L.) are dried grapes, consumed as energy dried fruits produced in the most region of the world. Raisins are a source of carbohydrates and contains large amount of iron, vitamins, calcium, potassium, flavonoids, polyphenols, glucose, fructose and minerals (Doymaz, 2006; Folts, 2002). Raisins are usually used in breakfast, cereals, dairy, bakery, in confectionery products and in nutritional bars (Ramos *et al.*, 2004; Yinshan *et al.*, 2017). Nowadays, raisins are produced from Thompson seedless grapes, introduced in California in 1862 by William Thompson (Rivero-Cruz *et al.*, 2008). In Pakistan, most of the grapes growing area is Baluchistan where it grows over an area of 13,000 ha with annual production of 49.0 thousand tones (Sajid & Ahmed, 2008). They are a source of fructooligosaccharides (fructans) acting as prebiotics helpful in colonic health and an important source of tartaric acid having beneficial role in intestinal function (Carughi *et al.*, 2012).

Natural occurrence of fungal contamination with its associated mycotoxins on dried fruits were investigated by several researchers (Abdel-Sater & Saber, 1999; Fernandez-Curz *et al.*, 2010; Azaiez *et al.*, 2015). During harvesting of grape, drying pressed, handling, transport and product exposure in markets make these raisins contaminated by different microorganisms (Magnoli *et al.*, 2003; Mandeel, 2005). *Aspergillus*, *Fusarium* and *Penicillium* are the major genera which attack and cause of mycotoxins production in food and dried fruits (Pitt, 2000). Aflatoxins are considered as the most toxigenic metabolites from mycotoxins classes produced mainly by *A. flavus* and *A. parasiticus* which cause diseases in human and animal (Kurtzman *et al.*, 1987). Mycological analysis of raisins showed contamination with fungi like *Aspergillus flavus*, *A. niger*, *A. sydowii*, *Penicillium citrinum*, *Alternaria* sp., *Trichoderma* sp and *Syncephalastrum* sp (Alhussaini, 2012). Saeed & Abdul-Rahman (2004) recorded 21 species belonging to 13 genera

from raisins including *Alternaria alternata*, *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *A. ochraceus*, *A. terreus*, *A. versicolor*, *Cladosporium cladosporioides*, *Fusarium oxysporum*, *Gibberella fujikuroi*, *Nectria haematococca*, *Nigrospora oryzae*, *Penicillium chrysogenum*, *P. citrinum*, *P. funiculosum*, *P. oxalicum*, *Rhizopus stolonifer*, *Scopulariopsis brevicaulis*.

Ultraviolet radiations has been known for many years to affect micro-organisms where UV-C is highly germicidal and used as sterilization of surfaces, water and air. UV-C radiations applied to air-conditioning systems helpful in reducing the incidence of *Cladosporium* spp., and *Aspergillus versicolor* (Levetin *et al.*, 2001). According to the report of Green *et al.* (2004), 35 and 54mJ cm² doses of ultraviolet radiation is necessary to inactivate most of the spores of *Aspergillus flavus* and *Aspergillus fumigatus*. Grape berries irradiating with UV-C produce no effect on filamentous fungi and even increased the incidence of yeasts and bacteria (Nigro *et al.*, 1998). Treatment time (100 s) from a distance of 3 cm and with 3800 V input is helpful in inactivation of *A. niger* spores in corn meal (Jun *et al.*, 2003).

Present study was carried out on mycoflora of raisins to find out its susceptibility profile as well as the effect of radiations on the preliminary incidence and trends of infection with pathogen during the storage period of raisins.

Materials and Methods

Sources of raisin samples: Fifteen dried samples of raisins were collected from the market of different cities of Pakistan like, Peshawar (1), Punjab, Baluchistan (1), Islamabad (1), Lahore (1), Rahim Yar Khan (1), Guddu (1), Karachi (6), Hyderabad (2) and Sukkur (1). These samples were collected from June, 2015 to December 2015 and brought to laboratory in a sterile polyethylene bag, sealed and placed in refrigerator (4°C) till mycoflora analysis.

Detecting mycoflora from raisins: Isolation of fungi on raisins was carried out following the rules of International Seed Testing Association (ISTA) by using Standard blotter, Agar plate and Deep freezing methods. About 400 raisins were tested in each method.

a) Standard blotter method: Raisins samples were first sterilized in 1 % sodium hypochlorite for 5 minutes and rinsed in several changes of sterile distilled water. These sterilized raisins were placed on three layers of moistened blotter paper (10 raisins per Petri dish). Control for each sample was also made in which raisins were not treated with sodium hypochlorite but was washed with sterilized distilled water. All the plates were incubated at temperature of $28\pm 2^{\circ}\text{C}$ for 7 days under 12h alternating cycle of artificial day light (ADL) and darkness (Anon., 1993).

b) Agar plate method: Same method as in standard blotter method was used except that the raisins after sterilization were placed on Potato Dextrose Agar (PDA) containing antibiotics (penicillin and streptomycin) instead of blotter paper (Anon., 1993).

c) Deep freezing method: Non sterilized and raisins after surface sterilization with sodium hypochlorite (5 minutes) were placed on three layers of moistened blotter paper and petri dishes were incubated for 24 hours, each at $28\pm 2^{\circ}\text{C}$ and -2°C followed by 5 days incubation at $28\pm 2^{\circ}\text{C}$ under 12 hours alternating cycle of artificial day light (ADL) and darkness (Anon., 1993).

Fungal identification: For identification of fungi, temporary slides were prepared from fungal colonies and observed under compound microscope (100, 400x). Fungi were identified using morphological characteristics like its colour, mycelial texture, pigmentation, spores characteristics (Barnett & Hunter, 1998; Booth, 1971; Ellis, 1971; Gilman, 1950; MycoBank, 2013; Nelson *et al.*, 1983; Raper *et al.*, 1965).

Treatment of raisins with ultraviolet radiation: Selected sample of raisins (which was highly infested with fungi) was subjected to ultraviolet radiations (UV-C) within the time period of 0, 5, 10 and 20 minutes and stored for 60 days at room temperature. Seeds were placed on Potato Dextrose Agar (PDA) poured plates (10 seeds/plate) at different time intervals of 0, 15, 30 and 60 days. Petri dishes were incubated for 5-7 days at $28\pm 2^{\circ}\text{C}$ under 12 hours alternating cycle of artificial day light (ADL) and darkness.

Data analysis: Data of infection % was subjected to analysis of variance (ANOVA) and mean were compared using Least Significance Difference (LSD) at 5 % probability level (Gomez & Gomez, 1984).

Results

Detection of fungi on raisins: Altogether 25 fungal species belonging to 15 genera were recorded from raisins by using ISTA (International Seed Testing Association) techniques (Table 1). Sixteen species of 9 fungal genera

were isolated through agar plate method, 16 species of 11 fungal genera were isolated by using blotter method while deep freezing method yielded 19 species belonging to 11 genera. Most dominant fungi in all the three methods were *Aspergillus niger* ($p<0.05$) followed by *A. flavus* and *Penicillium chrysogenum*. Deep freezing method favoured the growth of pathogenic fungi like *Fusarium oxysporum* ($p<0.05$), *Scopulariopsis acremonium*, *Chaetomium indicum* ($p<0.01$), and *Phoma* species. *A. niger* was found to have highest infection rate, showing almost similar infection in sterilized and non-sterilized condition. Species of *Mortierella* and *Phoma* were more frequently isolated in non-sterilized condition in only deep freezing method ($p<0.001$). Only one sample was found to be contaminated with *F. oxysporum*. Deep freezing method was found best for the isolation of fungi followed by standard blotter method. Samples of raisins from the areas of Lahore, Islamabad and Karachi, respectively were found to be highly infected with fungi. Species of *Aspergillus* and *Penicillium* were the most dominant fungi. Surface sterilization of raisins with 1 % sodium hypochlorite had reduced the incidence of storage fungi.

Mycoflora of raisins during storage: Raisins were treated with UV radiation with different time duration of 0, 5, 10 and 20 minutes (storage period 0 day and after 15, 30 and 60 days) showed an interesting pattern of fungi isolated by agar plate method. At 0 day, UV radiation showed heavy infection of *A. niger* only and raisins showed greater infection percentage. Infection percentage of *A. niger* in control was highest that was 78% followed by 58% of 5 minutes treatment, 38% in 10 minutes and 30% in 20 minutes of UV treatment. Infection of *A. niger* was reduced with increased in UV treatment time. After 15 and 30 days of storage period *A. niger* and *Penicillium* spp., were observed. After 60 days of storage period, raisins were infected with *Penicillium* and *Aspergillus* spp (Table 2).

Discussion

Fifteen samples of raisins collected from local vendors of different cities of Pakistan for the investigation of mycoflora by using ISTA techniques. Total number of 25 fungal species belonging to 15 genera were isolated. Isolation of fungi by using blotter, agar plate and deep freezing methods as recommended by ISTA (Anon, 1993) revealed that deep freezing method was best among the three. Similar results were also reported on *Pinus gerardiana* by Bilgrami & Ghaffar (1993), Niaz & Dawar (2009) on *Zea mays*. Deep freezing method was the best because *Aspergillus niger* and *Penicillium* species grew superficially on the other two methods which effected the number of fungi isolated on blotter paper and agar plate method. 19 species with 11 genera were isolated by deep freezing method, agar plate methods yielded 16 species belonging to 9 genera and 16 species belonging to 11 genera were isolated by blotter method. Deep freezing method was also found to be best for the isolation of slow growing seed borne fungi namely *Drechslera* spp., *Fusarium* spp., *Penicillium* spp., *Nigrospora oryzae*, *Macrophomina phaseolina*, *Alternaria alternata*, *Syncephalastrum racemosum* (Mathur *et al.*, 1975).

Table 1. Mycoflora of raisins (*Vitis vinifera* L.) isolated by ISTA technique.

Name of fungi	Sterilized fruits						Non-sterilized fruits					
	Blotter method		Agar plate		Deep freezing		Blotter method		Agar plate		Deep freezing	
	NSI	I % ± SD	NSI	I % ± SD	NSI	I % ± SD	NSI	I % ± SD	NSI	I % ± SD	NSI	I % ± SD
<i>Alternaria alternata</i>	-	-	-	-	1	0.13 ± 0.13	-	-	-	-	-	-
<i>Aspergillus clavatus</i>	-	-	-	-	1	0.26 ± 1.03	-	-	1	0.80 ± 3.09	1	0.26 ± 1.03
<i>Aspergillus carneus</i>	-	-	1	0.20 ± 0.57	-	-	-	-	-	-	-	-
<i>Aspergillus flavus</i>	5	4.06 ± 11.90	5	2.00 ± 4.34	4	2.6 ± 10.51	5	3.66 ± 9.33	5	1.60 ± 1.39	7	3.26 ± 8.83
<i>Aspergillus fumigatus</i>	3	0.66 ± 3.21	2	0.20 ± 0.42	2	0.6 ± 1.59	2	0.26 ± 0.77	1	0.06 ± 0.25	1	0.20 ± 0.75
<i>Aspergillus niger</i>	12	49.93 ± 27.63	12	47.5 ± 36.32	11	38.4 ± 34.02	15	64.53 ± 37.58	15	64 ± 37.46	11	53.06 ± 38.67
<i>Aspergillus oryzae</i>	5	3.73 ± 14.81	-	-	2	0.13 ± 0.34	3	3.8 ± 9.20	-	-	4	4.33 ± 13.84
<i>Aspergillus sclerotiorum</i>	-	-	1	0.13 ± 0.53	1	0.2 ± 0.75	-	-	-	-	1	0.06 ± 0.25
<i>Aspergillus terreus</i>	-	-	-	-	-	-	-	-	-	-	1	0.06 ± 0.25
<i>Aspergillus ustus</i>	2	0.53 ± 4.24	2	0.66 ± 2.39	3	3.46 ± 10.97	2	2 ± 5.43	-	-	3	2.46 ± 6.28
<i>Aspergillus wentii</i>	1	0.13 ± 0.51	1	0.06 ± 0.25	1	0.06 ± 0.25	-	-	-	-	-	-
<i>Chaetomium indicum</i>	1	0.06 ± 0.25	-	-	1	0.06 ± 0.25	-	-	-	-	1	0.2 ± 0.77
<i>Fusarium oxysporum</i>	1	0.06 ± 0.25	-	-	1	0.06 ± 0.25	-	-	-	-	1	0.06 ± 0.25
<i>Humicola fuscoatra</i>	1	0.20 ± 0.77	-	-	-	-	-	-	1	0.13 ± 0.51	-	-
<i>H. grisea</i>	-	-	-	-	1	0.06 ± 0.25	-	-	-	-	-	-
<i>Monilia</i> sp.	-	-	-	-	1	0.06 ± 0.25	1	0.06 ± 0.25	-	-	1	0.20 ± 0.77
<i>Mortierella</i> sp.	-	-	-	-	1	0.26 ± 1.03	-	-	1	0.46 ± 1.80	1	0.20 ± 0.77
<i>Mucor mucedo</i>	1	0.53 ± 2.06	2	0.46 ± 1.24	-	-	-	-	-	-	-	-
<i>Myrothecium roridum</i>	-	-	1	0.4 ± 1.54	1	0.06 ± 0.25	-	-	-	-	-	-
<i>Penicillium chrysogenum</i>	5	6.53 ± 17.29	4	3.26 ± 9.74	5	9.73 ± 10.57	3	2.86 ± 8.29	6	5.8 ± 22.35	4	3.6 ± 9.00
<i>Phoma</i> sp.	1	0.06 ± 0.25	-	-	-	-	1	0.2 ± 0.25	-	-	-	-
<i>Rhizopus oryzae</i>	2	0.2 ± 1.05	4	11.2 ± 25.35	-	-	-	-	4	2.06 ± 6.40	-	-
<i>Scopulariopsis acremonium</i>	1	0.06 ± 0.25	-	-	1	0.06 ± 0.25	-	-	-	-	1	0.2 ± 0.77
<i>Syncephalastrum</i> sp.	-	-	1	0.26 ± 1.03	-	-	-	-	1	0.4 ± 1.54	-	-
<i>Verticillium</i> sp.	1	0.66 ± 2.58	-	-	1	0.33 ± 1.29	1	0.46 ± 1.80	1	0.2 ± 0.77	1	0.73 ± 2.84

NSI = Number of samples infected; I% = Infection %; SD = Standard deviation

Table 2. Effect of Ultraviolet (UV-C) radiation on mycoflora of raisins (*Vitis vinifera* L.).

Fungi	Duration of UV-C treatment (minutes)			
	0	5	10	20
	I% ± SD	I% ± SD	I% ± SD	I% ± SD
0 DAY				
<i>Aspergillus niger</i>	78 ± 2.94	58 ± 2.28	38 ± 0.83	30 ± 1.00
15 DAYS				
<i>Aspergillus niger</i>	50 ± 2.34	80 ± 2.82	34 ± 1.67	74 ± 2.30
<i>Penicillium chrysogenum</i>	44 ± 0.89	24 ± 2.88	78 ± 1.78	50 ± 1.00
30 DAYS				
<i>Aspergillus niger</i>	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00
<i>Penicillium chrysogenum</i>	-	-	2 ± 0.44	-
60 DAYS				
<i>Aspergillus niger</i>	80 ± 2.73	68 ± 3.70	44 ± 4.82	38 ± 2.48
<i>A. oryzae</i>	-	12 ± 2.68	-	-
<i>Penicillium chrysogenum</i>	6 ± 0.89	-	-	-

I% = Infection %; SD = Standard deviation

Aspergillus was the most dominant genus among the isolated fungal species followed by *Penicillium* species in all samples. *Aspergillus* species was isolated from 79.5-90 % of contaminated fruits and vegetables (Peter *et al.*, 1990; Abdel-Sater & Saber 1999). Zohri & Abdel-Gawad (1993) found that *Penicillium* was the most predominant genus isolated from dried apricots, figs, prunes and raisins. In the present result, 11 *Aspergillus* species were isolated of which *A. niger* and *A. flavus* were the most prevalent species followed by *A. fumigatus*, *A. oryzae* and *A. ustus*. Remaining species were less frequently isolated while *A. candidus* and *A. sclerotiorum* were isolated from only one sample of dried raisins. Youssef *et al.* (2000) recorded same results on 100 samples of raisins collected from different markets of Egypt. Sample of Islamabad showed the high contamination with *Penicillium* spp and *Aspergillus wentii* that was also reported previously in *Pinus gerardiana* by Bilgrami & Ghaffar (1993). The main genera that attack and produce mycotoxins in food and dried fruits are *Aspergillus*, *Fusarium* and *Penicillium* (Pitt, 2000). *Penicillium* was highly encountered in all samples of raisins. Similar results were also obtained in cashew nut by Alhussaini (2012). Similar species of *Penicillium* was also reported in Gaborone, Botswana by Khare *et al.* (2013). Contamination of *Aspergillus* and *Penicillium* species suggest that most of the fungal invasion happened during storage where water activity and moisture content in the substrate was lower than those in the field (Pitt & Hocking, 2009). Similar findings were also reported by Alghalibi & Shater (2004) in which *Aspergillus* and *Penicillium* were isolated in higher frequency from different types of dried fruits. *Rhizopus* was also among the predominant genus and occupied most of the infected area of raisins. The same situation was also illustrated by Alghalibi & Shater (2004) that *Rhizopus stolonifer* was the second most common fungus isolated from dried raisins in Yemen. It occurred in 30% of the samples comprising 10.7 % of total fungi in dried raisins. It was not recorded by Abdel-Sater & Saber (1999) in their investigation and

isolated in low frequency from samples of dry raisins. The remaining fungal genera and species were less frequently isolated from the samples of dried raisins.

UV-C irradiation plays a major role in the selection of particular fungi that dominate the mycobiota of drying grapes. Treatment of raisins with ultraviolet radiation was also practiced to find out its significance on the occurrence of mycoflora on raisins and it worked in some instances but the *Aspergillus niger* appeared to be the most persistent fungus. However, it is not much surprising as the spores of *A. niger* are resistant to sunlight and UV radiation (Romero *et al.*, 2005). The second most dominating genus was *Penicillium* as previously reported that they remained in dried grapes in intense sunlight (Romero *et al.*, 2005; Belli *et al.*, 2004; Valero *et al.*, 2005). Beside *Penicillium*, some other fungi like *Aspergillus niger*, *Alternaria alternata*, *Cladosporium* spp., *Arthrimum phaeosporum* were also prevalent after solar exposure (Ulevicius *et al.*, 2004).

Grape raisins have become an important commodity in Pakistan. Importance of mycotoxins in food and feed has attained much priority in Pakistan. Many quests for better ways to control the contamination of aflatoxins, in particular and other mycotoxins, in general, during the last ten years, have given a boost to the food and feed sectors in Pakistan. Raisins are used locally in various delicious food recipes and dessert dishes and often are used for direct consumption. In present study, *Aspergillus niger*, *A. flavus* and *Penicillium chrysogenum* isolated from the raisins are also the major mycotoxins producers where *A. flavus* produced significant quantities of aflatoxins (Klich, 2007). However, Samson *et al.* (1995) observed that *P. chrysogenum* is a major producer of a wide range of toxic compounds which are hazardous to human health. Species of *Aspergillus*, *Mucor*, *Penicillium*, *Alternaria* and *Rhizopus* causing contaminants during raisins harbour and these fungi produced mycotoxins during consumption of raisins sold in Pakistan. Incidences to these fungi can be reduced by improving the storing packaging and retailing practices in terms of sugar and moisture content with relation to the geographical and climatic factors.

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