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OCCURRENCE AND DISTRIBUTION OF VEGETABLES SEED-BORNE MYCOFLORA IN PUNJAB PAKISTAN

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A B S T R A C T

Vegetable seeds are carrier of mycoflora inciting bulk of diseases, responsible for quality and yield losses. Majority of microbes are saprophyte and few are potential pathogen for the crop. In this research seed mycoflora were isolated by using standard blotter paper and agar plate technique. Major vegetables of summer and winter seeds were collected from regional local markets of Punjab province. Among these 15 fungal genera and 18 different species were identified. According to results highest incidence of fungal pathogens were *Aspergillus niger, A. flavus, Penicillium camemberti* and *Bipolaris* spp. and low incidence of *Stemphylium* spp., *Cladosporim* spp., *F. semitectum, Curvularia lunata, Trichoderma* spp., *Rhizopus nigricans, Paecilomyces lilacinus, Fusarium oxysporum, Drechslera australiensis, Ascochyta* spp., *A. fumigatus, Rhizoctonia* spp., *Alternaria alternata* and *Chaetomium globosum.* The agar plate method was found to be the most suitable technique for detection of seed-borne fungi in vegetable seed. The results of this study could be helpful for management of seed borne fungal pathogens of different vegetables. Our findings may also helpful for seed treatment before sowing with appropriate fungicides to overcome the losses caused by seed borne fungi.

Keywords: Saprophytes, Pathogenic, Detection, Isolation, Identification, Purification

INTRODUCTION

Vegetables are secondary food source of humans and rich source of nutrients, proteins, carbohydrates, minerals, fibers and vitamins essential for health (Sonni, 2002). In 2002-2003 vegetables are cultivated in Pakistan on an area of 0.22 million hectares which produce 2.88 million tons' vegetables. Exports of vegetables increased 39% during physical year of 2007-2011. Climate of Pakistan is very suitable for production of different vegetables. Among different vegetables bitter gourd (*Momordica charantia*), sweet gourd (*Cucurbita moschata*), okra (*Ablemochus esculentus*), pumpkin (*Cucurbita moschata*), bottle gourd (*Lagenaria siceraria*) and squash (*Cucurbita pepo*) were broadly cultivated and in case of winter vegetables carrot (*Daucus carrota var sativa*), turnip (*Brassica rapa*) and reddish (*Raphanus sativus*) were most cultivated vegetables in Pakistan described by (Ali and Kumar, 2000).

In Pakistan depletion of healthy seeds is very alarming especially in Punjab region. The share of vegetables seed is 72% in the Punjab province. In Punjab different vegetables pea, summer squash, carrot, okra, chilies, potato, pumpkin, bitter gourd, turnip, cauliflower and bottle gourd which have great importance in Pakistan reported by (Bakhsh, 2007).

Among different plant pathogen transporting agents' seeds are major contributors that covers long distances without any hurdle. The dispersal of diseases through seeds from one region to other region is well recognized (Agarwal and Sinclair, 1996). Seed-borne pathogens are involved in seed rottening during germination and seedling mortality leading to poor crop stand (Khalid *et al.*, 2001), plant

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growth reduction and low yield of crops (Kubiak and Korbas, 1999; Dawson and Bateman, 2001). Due to contamination of seeds initiation of local infection that reduce the germination rate, abortion of seed, seed rot, stunted growth, viability, and permeability. The main root rotting fungi that cause heavy losses to crops are *Phytophthora* spp., *Verticillium* spp. *Rhizoctonia* spp., *Pythium* spp., and *Phytophthora* spp. The fungal attack increased due to poor storage that causes discoloration, decaying of seed and mycotoxin production (Logrieco *et al.,* 2003). Thus in present study we concentrated on isolation, identification and purification of vegetables seed borne fungi existing in different districts of Punjab, Pakistan.

MATERIALS AND METHODS

Collection of seed samples: The seed samples were collected according to their area of production from different local markets, corporations, seed stores and research centers from Punjab province during winter and summer 2013-15 (Table 1). All seeds were categorized and preserved in polythene bags and stored according to dispensing conferring method (Yuan *et al*., 1997). In each lot 400 seeds were separated and used to check the pathogen infection by using blotter paper and agar plate methods (ISTA, 1993). All experiments were conducted in Department of Plant Pathology research area University college of Agriculture Sargodha.

Table 1. Sources and localities for seed samples of various vegetables.

Name of vegetable	Botanical name	Family	Summer/winter	Place of collection
Bitter gourd	*Momordica charantia*	Cucurbitaceae	Summer	Bhakkar
Bottle gourd	*Lagenaria siceraria*	Cucurbitaceae	Summer	Multan
Carrot	*Daucus carrota* var *sativa*	Apiaceae	Winter	Vehari
Cucumber	*Cucumis sativus*	Cucurbitaceae	Summer	Multan
Okra	*Abelmochus esculentus*	Malvaceae	Summer	Mianwali
Pea	*Pisum sativum*	Fabaceae	Winter	Narrowal
Pumpkin	*Cucurbita moschata*	Cucurbitaceae	Summer	Narowal
Radish	*Raphanus sativus*	Brassicaceae	Winter	Sargodha
Summer squash	*Cucurbita pepo*	Cucurbitaceae	Summer	Chiniot
Turnip	*Brassica rapa*	Brassicaceae	Winter	Sargodha

Agar plate method: Potato dextrose agar medium was prepared for fungal growth and sterilized at 121°C for 20 minutes at 15 psi pressure. After sterilization media cooled until mild hot and poured 15 ml in each Petri dish having 90 cm diameter. Seeds were surface sterilized with 1% NaOCl solution one and half minute and give three washes in sterilized double distilled water. After surface sterilization seeds were placed in Petri dishes containing media and incubate at 22 °C ± 1 °C for 5 to 7 days in an incubator. All processes were carried out under laminar flow chamber to maintain hygienic condition.

Standard blotter paper technique: Evaluation of seed borne mycoflora was done by using standard blotter paper technique. In this technique sterilized blotter paper was used in Petri dishes in spite of any medium. The 5-10 surface sterilized seeds were transferred in Petri dishes containing wet blotter paper in three layers and incubate at 25°C and maintain light and dark conditions (Anonymous, 1996). After germination of fungal mycoflora all fungal colonies were purified and observed under stereo microscope. The bud tip of each fungus was isolated and purified on other plates containing media for further identification. Each fungus was identified according to their physical and morphological properties by using illustrated genera (Nelson *et al*., 1983; Booth, 1971; Raper and Fennel, 1965). All treatments were replicated three times. Percentage frequency of seed borne mycoflora was estimated by using formula:

$$\text{Percentage mean frequency} = \frac{\text{No. of infected seed}}{\text{Total number of seed}} \times 100$$

Isolation and purification of seed-borne mycoflora: Isolation was done on the basis of tissue symptoms to find out the association of specific mycoflora by taking hyphal bud tip or single spore from growing colony on seeds and purified on alternate Petri dishes containing PDA media.

Single spore technique: In case of single spore technique, serial dilutions of spore suspension from seven days old culture were made in sterilized distilled water until a solution containing 10-15 spores/ml was achieved. One mL of this diluted spore suspension was poured in Petri dish containing two percent plain agar autoclaved for 15 minutes under aseptic conditions. Spore suspension was evenly distributed by tilting the Petri plate in various directions. After few minutes,

excess suspension was removed from Petri-dishes. Inoculated Petri dishes were incubated at 24 ±2°C for 24 hours. Germinating single spore was located and marked under the microscope and transferred on 2 percent PDA slants, aseptically. Inoculated slants were subsequently allowed to grow and sporulate.

Hyphal tip method: The method is same as described previous except that in its place of single spore, hyphal tip was marked and transferred on two per cent PDA slants.

Identification: Identification of isolated fungi was done by using synoptic key (Mathur and Kongsdal, 2003).

Statistical Analysis: Statistical analysis of all experimental data was done by using MSTAT statistical software.

RESULTS

The purpose of this research was to evaluate the presence of mycoflora on vegetables seeds. Identification of different fungi was done on the basis of morphology of fungal colony, spore and hyphal structure under compound microscope according to illustrated genera of fungi. There are 15 genera and 18 fungal species were identified on the basis morphology (Table. 2).

Table 2. Identification of all isolated fungi on the basis of Morphology

Fungi Name	Colony colour/Shape	Spore /Conidia	Hyphal stucture
Aspergillus niger	Date brown with white to cream thick mat of floccose mycelia at the edge	Conidia were biseriate and globose in shape	Fusiform shaped
A. fumigatus	Greenish grey with colorless mycelia	Rod shaped conidia	Filamentous hyphae
A. flavus	Yellow green with white mycelia at the edges	The conidia were rough	Septate and hyaline with thread like branching
Alternaria alternata	Colonies were brown segmented mycelia	Solitary apical spores	Muriform shaped hypahe
Bipolaris spp.	Black, velvet colonies and mycelium practically absent	Black and shiny conidia	Hyaline and pseudosepta
Chaetomium globosum	Colonies were cottony appearance with brown to blackish mycelium	Unidentifiable conidia	Football shaped
Cladosporim spp.	Long, branching filamentous structure	Ovoid, oblong, spherical and lemon-shaped	Long filamentous
Fusarium oxysporum	White to purple mycelium with distinct orange sporodochia	Smooth or rough walled	Fusiform, slightly curved and pointed at the tip
F. semitectum	Mostly straight with aerial mycelium	Simple or branched	Hyaline and septate
Paecilomyces lilacinus	Colonies were faint violet or mauve colouration which may change into a reddish grey colour	Long chain conidia	Hyaline and smooth walled hyphae
Penicillium camemberti	Colonies form a hard, white crust structures of mycelium	Rough and smooth	Thread-like hyphae
Rhizoctonia spp.	At initial stage colonies were colorless but become brown at maturity	Irregular shaped	Separated hyphae
Rhizopus nigricans	A unicellular to a dimorphic or filamentous appearance	Unidentifiable conidia	Filamentous, branching and generally lack cross walls
Stemphylium spp.	Colonies were brownish to black in color with suede to cottony surface	Spore has a transverse septation	Dematiaceous hyphae
Trichoderma spp.	Colonies were transparent or whitish in colour	Compact or loose tufts	Highly branched
Curvularia lunata	Colonies were brown to black colour, hairy, velvety or woolly in shape	Smooth texture conidia	3 septa and 4 cells with curved appearance
Drechslera australiensis	A gray to dark blackish brown colour mycelium	Straight or cylindrical	Solitary, flexible and septate
Ascochyta spp.	Brown to black colour mycelium	Pear shaped conidia	Varied shaped

Results showed that both techniques confirmed the dominance of saprophytic fungi which were *Rhizopus* spp.*, Aspergillus* spp.*, Curvularia* spp.*, Penicillium* spp. and *Chaetomium* spp. The plant pathogenic fungi *Drechslera* spp.*, Bipolaris* spp.*, Macrophomina* spp.*, Ascochyta* spp. and *Fusarium* spp were also dominant in few seeds.

General prevalence of fungi was as following in the table.

S.No.	Vegetable	Overall fungal isolates	Dominant fungus
1	Carrot	*A. niger*, *A. fumigatus*, *A. flavus*, *A. alternata*, *C. globosum*, *Cladosporium* spp., *P. lilacinus*, *P. camemberti*, *Rhizoctonia* spp.	*Bipolaris* spp., *Curvularia lunata*
2	Cucumber	*A. niger*, *A. fumigatus*, *A. flavus*, *Bipolaris* spp., *P. camemberti*, *Rhizoctonia* spp., *Stemphylium* spp.,	*Drechslera australiensis, Ascochyta* spp.
3	Okra	*A. niger*, *A. flavus*, *A. alternata, C. globosum*, *F. oxysporum, P. camemberti*, *Rhizoctonia* spp., *R. nigricans*, *Trichoderma* spp.,, *C. lunata*	*A. niger, A. alternata*
4	Pea	*A. niger*, *A. flavus*, *Bipolaris* spp., *C. globosum*, *Cladosporium* spp., *F. oxysporum*, *P. camemberti*, *D. australiensis*, *Ascochyta* spp	*A. niger*, *A. flavus*, *F. oxysporum*
5	Bottle guard	*A. niger*, *A. flavus*, *Bipolaris* spp., *Cladosporium* spp., *F. semitectum*, *P. camemberti, R. nigricans, D. australiensis*	*A. niger*, *A. flavus*, *Bipolaris* spp., *R. nigricans*
6	Summer squash	*A. niger*, *A. flavus*, *A. alternata*, *Bipolaris* spp, *C. globosum*, *P. lilacinus*, *P. camemberti*, *Stemphylium* spp., *C. lunata*	*A. alternata*, *A. niger*, *P. lilacinus*
7	Turnip	*A. niger*, *A. fumigatus*, *A. flavus*, *Bipolaris* spp., *F. oxysporum*, *F. semitectum*, *P. lilanicus*, *P. camemberti*, *R. nigricans*, *T. harzianum*	*Ascochyta* spp. *A. flavus, F. oxysporum, C. lunata*
8	Reddish	*A. niger*, *A. fumigatus*, *A. flavus*, *A. alternata*, *Bipolaris* spp., *C. globosum*, *F. oxysporum*, *F. semitectum*, *P. camemberti*, *Rhizoctonia* spp,	*Ascochyta* spp. *A. flavus, Bipolaris* spp., *C. lunata*
9	Pumpkin	*A. niger*, *A. fumigatus*, *A. flavus*, *A. alternata*, *Bipolaris* spp., *C. globosum*, *P. lilacinus, P. camemberti*, *Rhizoctonia* spp., *Trichoderma* spp.,, *D. australiensis, Ascochyta* spp	*R. nigricans*
10	Bitter gourd	*A. niger*, *A. fumigatus, A. flavus*, *A. alternata*, *Bipolaris* spp., *Chaetomium globosum*, *F. oxysporum*, *P. lilacinus*, *P. camemberti*, *C.lunata, Ascochyta* spp.	*Curvularia lunata*

The agar plate method was more affective then blotter paper method. In agar plate method fungal growth is higher than blotter paper and separation of different fungi on the base of morphology was easy than blotter paper method. Very high growth difference observed between these two scientifically proven methods. Due to high nutrient concentrations PDA medium boost the growth of fungal pathogen than blotter paper and highest fungal growth was at 25 °C. In agar plate method total isolates production were higher than blotter isolation technique (Table 3 and 4).

Table 3. Mycoflora of different vegetables seeds by using blotter paper method. Bi. G = Bitter gourd, Bo. G = Bottle gourd, Ca = Carrot, Cu = Cucumber, O = Okra, Pe = Pea, Pu = Pumpkin, R = Reddish, S. S = Summer squash and T = Turnip.

Sr. No.	Name of fungus	Frequency (%) of fungus incidence										Total freq.
		Bi. G	Bo. G	Ca	Cu	O	Pe	Pu	R	S. S	T	
1	*A. niger*	16.02	16.02	16.37	7.16	17.97	9.76	16.20	13.13	17.12	13.55	143.3
2	*A. fumigatus*	15.79	-----	16.25	6.59	-----	-----	16.32	14.12	-----	14.10	83.17
3	*A. flavus*	15.64	15.65	16.80	6.77	17.68	10.42	16.10	14.30	17.57	14.05	144.98
4	*A. alternata*	15.61	-----	15.78	-----	17.30	-----	16.13	13.47	15.83	-----	94.12
5	*Bipolaris* spp.	14.91	14.91	-----	6.61	-----	7.24	15.80	13.62	15.29	14.85	103.2
6	*C. globosum*	13.61	-----	15.06	-----	16.75	8.83	15.35	13.12	15.25	-----	97.97
7	*Cladosporium* spp.	-----	13.39	14.56	-----	-----	7.47	-----	-----	-----	-----	35.42
8	*F. oxysporum*	14.85	-----	-----	-----	17.42	8.25	-----	13.45	-----	13.40	67.37
9	*F. semitectum*	-----	14.94	-----	-----	-----	-----	-----	13.17	-----	13.51	41.62
10	*P. lilacinus*	12.36	-----	14.15	-----	-----	-----	13.73	-----	12.87	12.17	65.28
11	*P. camemberti*	13.97	13.57	15.20	5.74	15.27	17.49	14.80	12.80	14.32	12.76	135.92
12	*Rhizoctonia* spp.	13.25	-----	14.80	6.34	15.45	-----	14.32	12.65	13.75	-----	90.56
13	*R. nigricans*	-----	14.96	-----	-----	17.29	-----	-----	-----	17.68	14.29	64.22
14	*Stemphylium* spp.	-----	-----	-----	5.97	-----	-----	-----	-----	13.69	-----	19.66
15	*Trichoderma* spp.	-----	-----	-----	-----	15.75	-----	13.93	-----	-----	12.67	42.35
16	*C. lunata*	16.09	-----	15.55	-----	16.85	-----	-----	-----	15.35	-----	63.84
17	*D. australiensis*	-----	14.50	15.70	6.50	-----	17.91	15.00	-----	-----	-----	69.61
18	*Ascochyta* spp.	13.31	-----	13.41	5.50	-----	6.83	13.90	12.05	-----	12.10	77.10
	Total frequency	175.41	117.94	183.63	57.17	167.73	94.2	181.58	135.88	168.72	147.45	1429.71

Table 4. Mycoflora of different vegetables seeds by using agar plate method. Bi. G = Bitter gourd, Bo. G = Bottle gourd, Ca = Carrot, Cu = Cucumber, O = Okra, Pe = Pea, Pu = Pumpkin, R = Reddish, S. S = Summer squash and T = Turnip.

Sr. No.	Name of fungus	Frequency (%) of fungus incidence										Total freq.
		Bi. G	Bo. G	Ca	Cu	O	Pe	Pu	R	S. S	T	
1	*A. niger*	23.26	21.08	22.60	27.59	24.45	24.25	20.54	17.32	8.62	16.12	205.83
2	*A. fumigatus*	23.10	-----	23.20	28.48	-----	-----	22.70	15.08	-----	18.06	130.62
3	*A. flavus*	22.92	22.92	22.15	26.77	13.41	22.15	22.93	16.03	7.62	17.91	194.81
4	*A. alternata*	20.80	-----	19.70	-----	13.40	-----	21.06	13.35	8.46	-----	96.77
5	*Bipolaris* spp.	22.59	22.92	-----	25.55	-----	20.23	21.65	14.09	8.40	15.74	151.17
6	*C. globosum*	21.64	-----	17.58	-----	10.35	17.57	20.85	14.32	7.05	-----	109.36
7	*Cladosporium* spp.	-----	21.24	11.38	-----	-----	11.38	-----	-----	-----	-----	44.00
8	*F. oxysporum*	23.22	-----	-----	-----	12.03	20.97	-----	15.31	-----	17.87	89.4
9	*F. semitectum*	-----	22.91	-----	-----	-----	-----	-----	14.28	-----	16.92	54.11
10	*P. lilacinus*	18.80	-----	13.65	-----	-----	-----	19.83	-----	5.91	13.98	72.17
11	*P. camemberti*	22.15	22.15	14.43	26.93	12.37	14.43	20.55	15.12	7.48	14.59	170.2
12	*Rhizoctonia* spp.	21.58	-----	13.78	25.04	12.20	-----	20.30	13.47	9.19	-----	115.56
13	*R. nigricans*	-----	25.18	-----	-----	12.62	-----	-----	-----	8.07	17.36	63.23
14	*Stemphylium* spp.	-----	-----	-----	22.60	-----	-----	-----	-----	6.14	-----	28.74
15	*Trichoderma* spp.	-----	-----	-----	-----	11.22	-----	20.27	-----	-----	16.49	47.98
16	*C. lunata*	22.53	-----	22.13	-----	12.85	-----	-----	-----	9.27	-----	66.78
17	*D. australiensis*	-----	22.48	17.70	25.90	-----	17.70	20.95	-----	-----	-----	104.73
18	*Ascochyta* spp.	16.29	-----	12.53	23.68	-----	12.53	20.05	12.85	-----	13.07	111.00
	Total frequency	258.88	180.88	211.33	232.54	134.9	161.21	251.68	161.22	86.21	178.11	1856.96

DISCUSSION

Many fungal pathogens affect the vegetables production specially cucurbits (Abawi and Widmer, 2000). Pathogens cause various diseases such as Wilt, Rot, Damping off, Anthracnose, Phomopsis black stem, Phoma blight, Scab, Gummy stem blight, Downy mildew, Powdery mildews, Leaf spot and Leaf blight (Zitter *et al.,* 1996; Koike *et al.,* 2006). Seed is the first foundation brick for crop production. Healthy seed is a basic need to maintain and achieve plant populations for maximum yield. Among 16% annual crop losses, plant diseases were recognized as 10% and major contributor is contaminated seed (Fakir, 1983). In previous studies 15 genera and 29 fungal species were isolated from bitter gourd seeds in Pakistan using ISTA techniques (Sultana and Ghaffar, 2007).

Several seed borne fungi prevail on cucurbits including: *Botryodiplodia theobromae, A. alternata, C. lunata, Chaetomium* spp., *D. tetramera, F. equiseti, F. solani* and *F. moniliforme* on gourd seeds and on squash, watermelon, bitter gourd muskmelon and cucumber (Nair, 1982; Mathur, 1990). Several studies also reveal that rice seeds also infected with seed borne fungi (Khan *et al.,* 1988). In cucurbits blotter method was useful for detection of most infectious fungi (Begum and Momin, 2000; Elwakil and El-Metwally, 2001; Avinash and Ravishankar, 2013). Squash seed results confirm the previous recorded results in Pakistan (Rahim *et al.,* 2013). It is supposed due to the plenty of nutritional elements essential for fungal growth in the agar plate method. Soybean seeds infested with *Alternaria, Curvularia* and *Fusarium* coupled with isolation of *Aspergillus* spp. and *Penicillium* spp. were commonly found. These fungi cause seed bio deterioration of soybean seed (Chavan, 2011). The seed borne fungi reduce the germination rate, oil contents, carbohydrate, and protein; manipulate other biochemical changes in grains (Ijaz *et al.,* 2001). Fat and protein contents were reduced by *A. flavus, A. terreus, A. nigar, A. fumigates, A. versicolor* reduced the sugar contents (Chavan, 2011). The production of aflatoxin due to *Fusarium* spp. infestation cause devastative impact on seed germination and health (Ozcelik *et al.,* 1990; Frisvad and Thrane, 2004). *Fusarium* species are mostly seed borne or soil borne which cause root rot and damage seedlings and seeds (Anjorin *et al.,* 2008; Liu *et al.,* 2012). Generally, *Rhizoctonia, Phytopathora, Pythium* and *Fusarium* spp. under suitable environmental conditions cause seedling death and also kill the seeds before germination (Leslie *et al.,* 2005; Broders *et al.,* 2007). Our findings provide the base for seed handling

before sowing. The relationship of these saprotrophic and pathogenic fungi is well established according to previous reports (Fakhrunnisa and Ghaffar, 2006; Niaz and Dawar, 2009). Our findings also endorse the previous reports.

CONCLUSION

This effort provides the availability of seed standard in our markets that is very alarming. It is proved that seeds of vegetables are contaminated with different soil borne fungi that cause severe losses. Plant pathogenic fungi are prevailing in seeds and that ultimately transferred in farmer's fields which cause fields which cause losses in the form of poor germination and early disease spread. There is a big requirement to screen the seeds for healthy crop and maximum yield.

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