Journal of Applied and Natural Science 8 (3): 1229 - 1234 (2016)

JANS

Prevalence and morphological characterization of *Aspergillus* isolates of maize ear rot in Punjab

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Received: October 30, 2015; Revised received: April 08, 2016; Accepted: July 12, 2016

Abstract: Among six fungal species viz.*Aspergillus* spp., *Fusarium* spp., *Penicillum* spp., *Rhizopus* spp., *Trichoderma* spp. and *Alternaria* spp. isolated from maize ear rot samples collected from five different districts of Punjab, the incidence of *A. flavus* was highest (42.15%) followed by *Penicillium* spp. (20.75%). The maximum frequency of *A. flavus* (57.10%) was found in Hoshiarpur district, closely followed by Jalandhar. In all, thirty nine isolates of *Aspergillus* spp. (33 isolates of *A. flavus* and 6 isolates of *A. niger*) were characterized morphologically. Twenty isolates of *A. flavus* producing sclerotia were of L-type strains having sclerotia diameter >400 µm. Isolates of *A. flavus* produced yellowish green, dark green and light green colonies and isolates of *A. niger* produced dark black colonies. Sterigmata in all the isolates of *Aspergillus* spp. were of uniseriate type. Based on colony diameter and growth rate per day all the 39 isolates of *Aspergillus* spp. were grouped into fast, medium and slow growing categories. Based on multivariate cluster analysis, the isolates of *A. flavus* were grouped into three distinct clusters each having 13, 17 and 3 isolates respectively.In the present study, *Aspergillus flavus* was found predominantly associated species with the maize ear rot. Further, the morphological variation observed within *Aspergillus flavus* and *A. niger* indicated the need for proper surveillance and monitoring exclusively for the prevention of moulds in maize produce in Punjab before it reaches the consumer.

Keywords: *Aspergillus flavus*, *A. niger*, Ear rot, Isolate,Maize

INTRODUCTION

Ear rot is the most potentially damaging disease of maize crop (Gxasheka *et al*., 2015). Several fungal species viz: *Aspergillus*, *Fusarium* and *Penicillum* are associated with the maize grains in field as well as in storage. Ear rot disease results in reduced grain but the main loss from ear rot disease is due to the contamination of grain yield with mycotoxins which are a threat to safety of both humans and livestock (Bello *et al.*, 2012). The direct economic impact of fungal and mycotoxin contamination in maize grains results mainly from a reduction in marketable volume, loss in value in the national markets, inadmissibility or rejection of products by the international market, and losses incurred from livestock disease, consequential morbidity and mortality. The average economic loss due to mycotoxin contamination is estimated at approximately one billion dollars, with aflatoxins representing a large proportion of this loss in the United States (Amaike and Keller, 2011). Aspergillus ear rot of maize is predominately caused by *A. flavus* and is prevalent where drought conditions occurs and can lead to the accumulation of aflatoxins in grains (Nicholson *et al.*, 2004). Aflatoxicosis causes acute liver damage, liver cirrhosis, induction of tumors, impaired central nervous system, skin disorder and hormonal defects.

Morphological characterization is the primary tool for the identification of *Aspergillus* species. Although molecular methods continue to improve and become more rapidly available, microscopy and culture remain commonly used and essential tools for identification of *Aspergillus* spp. (McClenny, 2005). As with fungi in general, *Aspergillus* taxonomy is complex and ever evolving. The genus is easily identified by its characteristic conidiophore, but species identification and differentiation is complex, for it is traditionally based on a range of morphological features. Macromorphological features which are often considered include conidial and mycelial color, colony diameter, colony pigmentation, production of exudates and soluble pigments, presence of sclerotia and cleistothecia. Micromorphology characterization is mainly dependent on seriation, shape and size of vesicle, conidia and stipe morphology and morphology of cleistothecia and ascospores (Klich, 2002). The best quality foods produced in some nations are rejected for export because of aflatoxin levels exceeding the tolerable limit, resulting in millions of dollars in losses. Its major cause is the presence of moulds especially *Aspergillus flavus* asso-

ciated with grains at the time of harvesting or storage. Since crop and aflatoxins are of paramount importance to Punjab State and no studies have been conducted on presence of different fungal genera associated with maize ear rot in Punjab. Keeping this in mind, the present study was focused on prevalence of different fungal genera associated with maize ear rot in different maize growing areas of Punjab and to characterize species of *Aspergillus* morphologically.

MATERIALS AND METHODS

Sampling and isolations: Maize ear rot samples were collected from different maize growing districts of Punjab viz., Ludhiana, Jalandhar, Hoshiarpur, Gurdaspur and Kapurthala during *Kharif* 2013 and spring 2014. Isolations of fungal spp. associated with maize ear rot were made from the infected samples on potato dextrose agar (PDA) medium. Six fungal spp. *Aspergillus* spp., *Fusarium* spp., *Penicillum* spp., *Rhizopus* spp., *Trichoderma* spp. and *Alternaria* spp. were isolated from the diseased samples. The cultures thus obtained were purified and maintained on PDA slants for further studies.

Morphological characteristics of *Aspergillus* isolates: Thirty three isolates of *Aspergillus flavus* and six isolates of *A. niger* were characterized morphologically. A 5 mm mycelium disc was cut from the actively growing edge of 5 day old culture and placed on Petri dish containing 20 ml PDA and incubated at 25±2˚C. Colony color, pigmentation (Anonymous, 2015), type of sterigmata, growth rate and production of sclerotia were observed after 7 days of incubation. Observations on radial growth pattern were taken at every 24 hrs interval. Each isolate was replicated thrice. The diameter of conidia, size of conidiophore and sclerotia diameter were observed microscopically and measurements were taken using image analysis software connected to Lecia DM 3000 microscope.

Multivariate cluster analysis was done for both qualitative and quantitative cultural characters using the Unweighted Pair Group Mean Average (UPGMA) with statistical analysis tool PAST ver. 2.1.5 and dendrogram was constructed.

RESULTS

Prevalence of ear rot fungi: The data on frequency of occurrence of different microorganisms associated with maize ear rot in different districts of Punjab are presented in Table 1. The data indicated that maximum incidence of *A. flavus* (57.1%) and *Fusarium* spp. (14.8%) was found in Hoshiarpur district, closely followed by Jalandhar (54.5%). All six fungal species (*Aspergillus* spp., *Fusarium* spp., *Penicillum* spp., *Rhizopus* spp., *Trichoderma* spp. and *Alternaria* spp.) were isolated from Ludhiana samples. The maximum incidence of *A. niger* was observed in Ludhiana (9.9%) district while minimum in Hoshiarpur (7.1%) district. The frequency of *Penicillum* spp. (33.7%) and *Rhizopus* spp. (28.1%) was observed maximum in Gurdaspur and minimum in Hoshiarpur district.

Among all the 7 species (*A. flavus*, *A. niger*, *Fusarium* spp., *Penicillum* spp., *Rhizopus* spp., *Trichoderma* spp. and *Alternaria* spp.) associated with maize ear rot, the incidence of *A. flavus* (42.15%) was highest followed by *Penicillium* spp. (20.75%) and *Rhizopus* spp. (15.67%). The incidence of *Alternaria* spp. was lowest only up to 0.67 per cent (Fig. 1).

Morphological characterization

Qualitative characters: The isolates of *A. flavus* within themselves do not show much variation in colony color. Except Af 12, Af 13, Af 16, Af 19 and Af 30, rest all isolates were having yellow green colonies (Table 2). Out of these, four isolates Af 12, Af 13, Af 16 and Af 19 were having dark green colonies and one isolate Af 30 was having light green colonies. The pigmentation of all the isolates varied from creamish to creamish yellow. All isolates of *A. niger* were having dark black colonies with cream color pigmentation whereas An 34 had dark black colonies with whitish margin and yellow color pigmentation.

Based on the average colony growth, all the isolates of *A. flavus* and *A. niger* were categorized as fast (> 75 cm), medium (75-80 cm) and slow (< 80 cm) growing (Table 3). The data indicated that eight isolates (Af 2, Af 5, Af 9, Af 13, Af 18, Af 27, Af 30 and Af 33) of *A. flavus* were slow growing with average colony diameter of <75mm and average growth rate per day ranging from 9.3 to 10.6 mm. Six isolates (Af 1, Af 4, Af 11, Af 15, Af 31 and Af 32) had medium growth with average colony diameter in the range of 75-80 mm and average colony growth rate ranging from 10.7 to 10.9 mm per day. Rest of the isolates were relatively fast growing with average colony diameter of >80 mm and average colony growth rate of 11.0 to 12.7 mm per day. Similarly, in isolates of *A. niger*, three isolates (An 34, An 38 and An 39) were slow growing with average colony diameter of <75mm and average

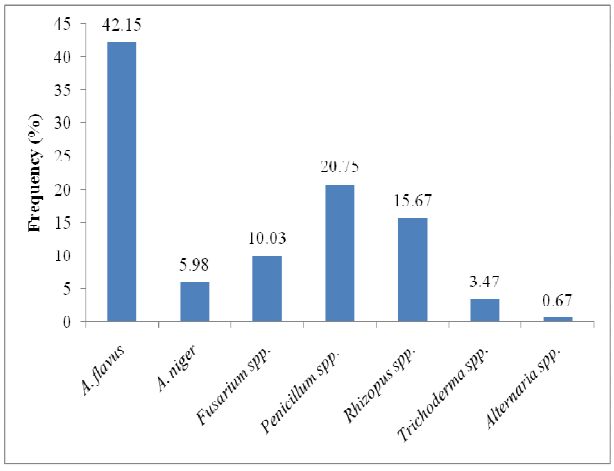

Fig. 1. *Prevalence of different microorganisms associated with ear rot of maize.*

Table 1. Frequency of occurrence of different microorganisms associated with maize ear rot in different districts of Punjab.

Microorganism	Frequency (%)				
	Ludhiana	Jalandhar	Hoshiarpur	Gurdaspur	Kapurthala
A. flavus	31.3	54.5	57.1	30.6	45.5
A. niger	9.9	0	7.1	0	9.1
Fusarium spp.	13.9	9.1	14.8	0	8.1
Penicillum spp.	15.7	18.2	14.3	33.7	27.3
Rhizopus spp.	17.7	18.7	7.1	28.1	0
Trichoderma spp.	5.9	9.1	0	0	0
Alternaria spp.	4	0	0	0	0

growth rate ranging from 5.5 to 9.5 mm per day. Only one isolate An 35 had medium growth with average colony diameter in the range of 75-80 mm and average colony growth rate of 9.6 to10.9 mm per day. Fast growing isolates, An 36 and An 37 have average colony diameter of >80 mm and average colony growth rate of 11.0 to11.6 mm per day.

Quantitative characters: All the isolates of *A. flavus* and *A. niger* produced abundant, single celled and round conidia while conidiophores have vesicles bearing conidia on uniseriate sterigmata. The data revealed that conidia diameter of *A. flavus* isolate Af 6 was maximum (30.7), whereas it was minimum (19.8) in Af 12 isolate (Table 2). The size of conidiophores of *A. flavus* isolates varied greatly in length and slightly in width. The mean length of conidiophores of *A. flavus* isolates varied from 227.5 to 1204.2 µm and mean width varied from 11.2 to 28.9 µm. Mean conidia diameter of *A. niger* isolates was in the range of 26.3 to 30.7µm, found minimum in An 38 and maximum in An 39 isolate. The mean length and width of conidiophores of *A. niger* isolates was in the range of 512.1 to 824.7 µm and 8.7 to 19.7 µm respectively. The isolate An 36 had minimum width and isolate An 39 had maximum width.

Out of 39 isolates, only 20 isolates of *A. flavus* produced sclerotia. Size of sclerotia varies from 747.3 to 1155.2 µm, found maximum and minimum in isolate Af 11 and Af 22, respectively. All the isolates producing sclerotia were of L-type having sclerotia diameter of >400µm.

Cluster analysis: Based on morphological characters, isolates of *A. flavus* were grouped into three clusters each having 13, 17 and 3 isolates respectively (Fig. 2). One isolate Af 33 from Kapurthala district out group cluster I with minimum growth rate per day (9.5 mm) and least conidiophores length (227.50µm). Cluster-II containing 17 isolates can be differentiated from the other two clusters on the basis of conidia size and growth rate (Table 4). The mean conidial diameter was minimum (28.2µm) and mean growth rate per day was found maximum (11.2) in cluster-II than other clusters. However size of conidiophores was more than cluster-I but less than cluster-III. Sclerotia diameter was also minimum (936.5) in cluster-II than cluster-III. Cluster-I can be differentiated from the other two clusters on the basis of sclerotia production. There was no sclerotia production in isolates grouped in cluster-I. Cluster-III containing 3 isolates had largest size of conidia (29.7µm), conidiophore (1059.1µm) and sclerotia

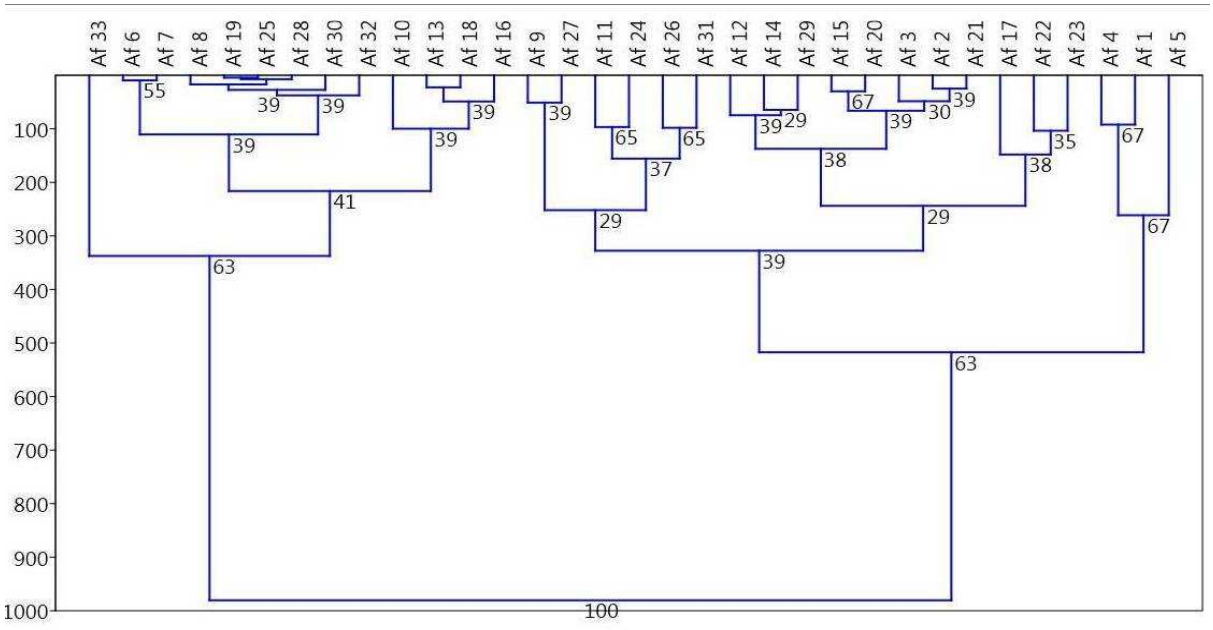

Fig. 2. *Dendrogram showing clustering of A. flavus isolates based on morphological characteristics.*

Table 2. Morphological characteristics of isolates of *Aspergillus* spp. collected from different maize growing districts of Punjab.

Isolate	Colony color	Pigmentation	Diameter of conidia (µm)*	Conidiophore size (µm)*		Diameter of sclerotia (µm)* at 5X
				Length	Width	
Af 1	Yellow green	Creamish	30.0 (26.3-34.6)	952.7 (855.7-1042.4)	17.6 (15.0-19.2)	1027.6 (913.2-1277.7)
Af 2	Yellow green	Creamish	28.3 (23.9-38.4)	588.3 (558.8-636.2)	23.1 (20.3-27.0)	898.9 (820.4-957.4)
Af 3	Yellow green	Creamish yellow	26.9 (23.4-31.3)	580.1 (544.8-615.5)	22.3 (18.1-24.5)	861.3 (806.6-912.0)
Af 4	Yellow green	Creamish	29.9 (21.9-38.1)	1020.5 (920.7-1082.8)	19.0 (15.2-24.8)	965.0 (915.3-1036.1)
Af 5	Yellow green	Creamish	29.0 (25.6-35.4)	1204.2 (1200.2-1208.2)	21.2 (19.2-23.5)	851.7 (775.7-938.8)
Af 6	Yellow green	Creamish	30.7 (27.2-33.0)	577.9 (566.5-589.4)	11.2 (8.5-14.4)	-
Af 7	Yellow green	Creamish yellow	24.5 (16.8-28.9)	572.3 (506.6-607.3)	15.8 (11.9-19.2)	-
Af 8	Yellow green	Creamish	27.5 (20.4-32.4)	453.7 (433.3-474.2)	17.1 (14.4-21.5)	-
Af 9	Yellow green	Creamish	30.4 (22.5-34.1)	428.5 (402.6-489.5)	15.6 (12.1-18.1)	1068.8 (1028.6-1121.6)
Af 10	Yellow green	Creamish	28.7 (23.1-38.9)	783.2 (780.5-785.9)	14.0 (12.1-16.8)	-
Af 11	Yellow green	Creamish	30.2 (26.3-33.4)	278.2 (217.4-339.4)	15.6 (12.1-19.6)	747.3 (708.6-807.3)
Af 12	Dark green with whitish periphery	Creamish	19.8 (12.8-32.3)	727.4 (696.1-756.5)	14.6 (12.1-17.3)	855.1 (778.7-971.6)
Af 13	Dark green with whitish periphery	Creamish	20.9 (23.4-31.3)	677.8 (610.7-717.6)	18.9 (15.2-22.4)	-
Af 14	Yellow green	Creamish	26.9 (13.8-33.3)	709.3 (685.6-762.2)	15.3 (11.9-18.8)	924.9 (788.1-1014.6)
Af 15	Yellow green	Creamish	24.0 (14.3-35.3)	644.6 (598.6-667.8)	22.1 (18.1-26.6)	914.8 (771.8-1041.9)
Af 16	Dark green with white periphery	Creamish	30.3 (21.9-40.6)	715.9 (677.0-750.2)	16.2 (14.2-19.6)	-
Af 17	Yellow green	Creamish	28.7 (22.9-36.9)	774.1 (757.1-787.4)	16.5 (12.8-19.2)	1118.8 (1071.8-1171.6)
Af 18	Yellow green	Creamish	29.0 (22.2-38.4)	657.3 (606.7-725.7)	14.1 (12.8-16.6)	-
Af 19	Dark green with white periphery	Creamish	30.4 (24.8-35.7)	471.7 (460.1-490.9)	12.4 (10.1-15.9)	-
Af 20	Yellow green	Creamish	29.0 (25.4-33.1)	637.8 (618.8-656.8)	22.3 (18.1-28.6)	886.0 (857.4-936.4)
Af 21	Yellow green	Creamish	30.0 (23.8-35.8)	574.6 (568.5-580.7)	23.1 (21.3-25.6)	919.6 (915.7-923.1)
Af 22	Yellow green	Creamish	29.35 (26.3-37.3)	594.7 (590.5-598.9)	19.7 (16.8-23.3)	1155.2 (1125.0-1225.0)
Af 23	Yellow green	Creamish	30.6 (23.8-36. 0)	667.45 (644.4-690.5)	20.4 (18.6-22.4)	1081.4 (982.3-1143.2)
Af 24	Yellow green	Creamish	29.8 (20.3-36.0)	276.23 (232.2-301.4)	14.3 (12.1-16.8)	843.9 (797.4-893.0)
Af 25	Yellow green	Creamish	29.0 (23.6-34.6)	473.4 (419.1-504.6)	16.1 (12.8-21.4)	-
Af 26	Yellow green	Creamish	29.6 (26.3-34.1)	414.1 (349.2-449.8)	13.4 (9.8-17.4)	802.2 (633.5-893.7)
Af 27	Yellow green	Creamish	28.8 (24.6-36.4)	455.7 (355.4-562.5)	13.0 (10.1-15.2)	1025.6 (1021.7-1030.6)
Af 28	Yellow green	Creamish	30.5 (23.4-41.5)	485.6 (403.8-590.5)	12.7 (7.5-16.8)	-
Af 29	Yellow Green	Creamish	26.2 (23.4-34.6)	773.0 (710.4-896.0)	21.8 (18.1-26.6)	916.2 (850.1-986.1)
Af 30	Light green	Creamish	29.2 (24.2-37.2)	491.5 (434.9-554.0)	23.5 (19.0-27.8)	-
Af 31	Yellow green	Creamish	29.9 (24.5-39.2)	400.9 (693.8-461.4)	17.8 (14.4-19.6)	899.3 (811.0-983.8)
Af 32	Yellow green	Creamish	29.7 (23.4-36.0)	433.7 (418.0-446.7)	15.6 (13.8-19.6)	-
Af 33	Yellow green	Creamish	29.1 (25.3-35.4)	227.5 (367.9-560.1)	28.9 (22.5-33.4)	-
An 34	Dark black with white margin	Yellowish	27.6 (23.0-31.7)	744.3 (700.9-787.6)	11.0 (9.8-12.9)	-
An 35	Dark black	Creamish	29.6 (23.9-35.8)	695.3 (612.6-778.1)	8.8 (6.7-12.9)	-
An 36	Dark black	Creamish	30.6 (23.9-38.1)	512.1 (478.7-545.5)	8.7 (7.7-9.7)	-
An 37	Dark black	Creamish	27.2 (22.0-31.0)	716.5 (688.1-742.6)	9.3 (8.0-11.9)	-
An 38	Dark black	Creamish yellow	26.3 (21.3-36.9)	824.7 (805.1-844.3)	9.6 (6.7-12.8)	-
An 39	Dark black	Creamish	30.7 (25.8-36.3)	695.3 (678.1-712.6)	19.7 (14.2-22.5)	-

Table 3. Categorization of isolates of *Aspergillus* spp. on the basis of their colony growth.

***Aspergillus* spp.**	**Isolate (s)**	**Colony diameter (mm)**	**Growth rate/ day (mm)**
A. flavus	Af 2, Af 5, Af 9, Af 13, Af 18, Af 27, Af 30, Af 33	<75	9.3-10.6
	Af 1, Af 4, Af 11, Af 15, Af 31, Af 32	75-80	10.7-10.9
	Af 3, Af 6, Af 7, Af 8, Af 10, Af 12, Af 14, Af 16, Af 17, Af 19, Af 20, Af 21, Af 22, Af 23, Af 24, Af 25, Af 26, Af 28, Af 29	>80	11.0-12.7
A. niger	An 34, An 38, An 39	<75	5.5-9.5
	An 35	75-80	9.6-10.9
	An 36, An 37	>80	11.0-11.6

Table 4. Morphological characters of different clusters of *A. flavus.*

Cluster	**Isolates**	**Conidia diameter (µm)***	**Size of conidiophore (µm)***		**Sclerotia diameter (µm)***	**Mean growth rate per day (mm)***
			Length	**Width**		
I	Af 33, Af 6, Af 7, Af 8, Af 19, Af 25, Af 28, Af 30, Af 32, Af 10, Af 13, Af 18, Af 16	28.5 (20.9-30.7)	540.1 (227.5-787.2)	16.7 (11.3-28.9)	-	11.1 (9.5-12.7)
II	Af 9, Af 27, Af 11, Af 24, Af 26, Af 31, Af 12, Af 14, Af 29, Af 15, Af 20, Af 3, Af 2, Af 21, Af 17, Af 22, Af 23	28.2 (19.9-30.6)	560.3 (276.2-774.1)	18.3 (13.1-23.2)	936.5 (747.3-1155.2)	11.2 (9.3-12.2)
III	Af 4, Af 1, Af 5	29.7 (29.0-30.0)	1059.1 (952.7-1204.0)	19.3 (17.7-21.3)	948.2 (851.8-1027.7)	10.7 (10.6-10.8)

*Mean of 50 observations and range is given in parenthesis- Sclerotia absent

(948.2µm) than cluster-I and cluster-II isolates. However mean growth per day was minimum (10.7mm/ day) in cluster-III than other two clusters. Isolate Af 5 (Ludhiana isolate) outgroup cluster-III with maximum conidiophore length of 1204.2µm.

DISCUSSION

Ear rot fungi are associated with maize grains in the field as well as under storage conditions. Out of six fungal species isolated, *A. flavus* was found predominantly associated with maize grains. Its frequency was found maximum in Hoshiarpur district of Punjab. These findings support the observations made by several workers (Atehnkeng *et al.*, 2008 and Abriba *et al.*, 2013). Atehnkeng *et al.* (2008) also found that the incidence of *Aspergillus* species was highest on maize grains and within *Aspergilllus*, *A. flavus* was the most commonly isolated species. Similarly, Abriba *et al* (2013) reported that most predominant genera associated with maize and other food grains was *Aspergillus.*
Morphological characteristics are the primary tools in the identification of various *Aspergillus* species. Despite originating from different districts, isolates showed similarities in their morphological and cultural characteristics. There was not much variation in colony color and pigmentation of *A. flavus* and *A. niger* isolates (Reddy *et al.*, 2010; Gautam and Bhadauria, 2012, Odhiambo *et al.*, 2013 and Baquiao *et al.*, 2013). However, there was significant difference among isolates with regard to colony diameter. Isolate of Jalandhar district was fast growing as compared to other districts. Least growth rate was observed in isolates of Ludhiana and Kapurthala district. Several workers (Kumar *et al.*, 2014, Navya *et al.*, 2014 and Amrita and Richa, 2014) have also reported significant differences in growth rate of *Aspergillus* isolates.
Observations on quantitative characters revealed that twenty isolates of *A. flavus* formed sclerotia and were of the L-strain type having sclerotia diameter of more than 400µm. Nayak *et al.*, (2014) and Nyongesa *et al.*, (2015) also reported that *A. flavus* produed sclerotia of L-strain type that is having sclertioal diameter more than 400µm. The size of conidiophores of *Aspergillus* spp. isolates varied greatly in length and slightly in width. Conidia diameter also shows slight variation in all the isolates. Variations in conidiophore length and conidia size of *A. flavus* and *A. niger* was also observed by Reddy *et al.* (2010).

Conclusion

The observations of present study showed that *A. flavus* was the most predominantly associated species with maize ear rot in Punjab. Its incidence was found maximum in Hoshiarpur, the major maize growing district of Punjab. The isolates of Jalandhar district were fast growing as compared to Kapurthala district. The present study contributed to the knowledge on diversity of *A. flavus* and *A. niger* associated with maize ear rot. Sclerotia producing isolates of *A. flavus* were of L-strain type having sclerotia diameter more

than 400µm. Though the clusters varied from place to place but high level of morphological and cultural diversity existed among the isolates *A. flavus* and *A. niger* .

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