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Exogenous factors affecting growth of *Aspergillus species*

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ABSTRACT

Aflatoxins are the most deleterious fungal metabolites in humans, animals and plants. Recently, more attention has been paid to the occurrence and growth of *Aspergillus* spp. In this study, the effects of pH, light, relative humidity (RH) and temperature on the growth of *Aspergillus spp.* were investigated using broth medium. Results revealed highest mean of dried mycelial weight (365.67mg) at pH 4.0. The highest spores per ml (8.243 × 107) at pH 5.0. Similarly, growth during darkness was higher on first day of incubation in *A. candidus* and *A. niger*, while *A. flavus* and *A. fumigatus* showed the highest growth under continuous light. Lower RH% (32.5%) favored only the growth of *A. niger*. However, the growth in other *Aspergillus* species was significantly increased by RH% (50.5%, 85.0% and 100%). At 40°C, only *A. fumigatus and A. flavus* showed significant (P<0.05) growth. There were significant differences in all the treatments (P<0.05). This proposes that these interacting environmental conditions impact significant effect on *Aspergillus* growth.

Key words: Aflatoxin, *Aspergillus,* light, pH, Relative humidity, Temperature.

INTRODUCTION

Aspergillus growth reduces the nutritional value of feed stuff by producing aflatoxin and allergenic spores (Naseer *et al*., 2016a, 2017b). In 1729, Antonio Micheli named genus *Aspergillus* for the first time and developed a major science, *i.e.* mycology. *Aspergillus species* affect food items, pharmaceutical products and other household commodities like wood, leather and textile goods. Pathogenic *Aspergillus spp*. produces aflatoxin cause numerous diseases like avian aspergillosis and bovine mycotic abortion. (Medina *et al*., 2014).

pH, light and temperature are important landmarks to understand the ecology of fungal mycotoxigenic spp. (Ahmed *et al*., 2009). pH effects fungal growth directly effecting on cell surface or indirectly by effecting nutrient availability. Required pH for growth ranges from 3.0 to 8.0, with optimal level of around 5.0 under the satisfactory nutrient status. Generally, *Aspergillus* spp. are more tolerant to alkaline pH as compared to others (Pardo *et al*., 2006). Molds react to light radiations in different ways, depend upon the irradiance and wavelength of the incident photons that strike the cells (Fuller *et al*., 2013, 2015). Moderate exposure to visible light (400-700 nm) encourages production of conidial spores, photo-protective pigments and harmful secondary metabolites in aflatoxin (Olmedo *et al*., 2013; De Menezes *et al*., 2015). Exposure to light can also render mold structures such as mycelia and conidia wth stress resistance (Fuller *et al*., 2013; De Menezes *et al*., 2015). Relation between light-sensing, virulence in animal- and plant-pathogenic fungi and fungal pathogenicity have also been reported (Yu *et al*., 2013; Cheng *et al*., 2014).

Climatic models have proposed a marked reduction in precipitation during summer and increase in temperature, ultimately resulting in episodes of drought. Interestingly, the climate change has an impact on economically important crops by contaminating with mycotoxin. (Magan *et al*., 2011; Wu *et al*., 2011). Environmental conditions may influence the association between various mycotoxigenic species and mycobiota (Magan *et al*., 2010; Paterson and Lima, 2012).

The main objective of the present study was to evaluate the *in-vitro* growth of *Aspergillus species* associated with various levels of pH, temperature, relative humidity and different regimes of light to comprehend sporulation and growth in the feed, so as to control the quality of feed and feed stuff from formulation to storage.

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MATERIALS AND METHODS

Collection of Fungi

Aspergillus parasiticus was obtained from the Department of Microbiology, University of Veterinary and Animal Sciences Lahore. The fungus was grown by sub-culturing on potato dextrose agar (PDA) in Petri dishes.

Preparation of potato dextrose broth media of varying pH levels

Potato dextrose broth (PDB) was prepared as described by Ayodele *et al.,* (2007). 300g of peeled Irish potato (*Solanum tuberosum*) was boiled in two fifty (250) ml of distilled water and strained through a muslin fabric. Twenty (20) gram of glucose and 0.05g of chloramphenicol were added to the filtrate and volume was made up to one liter by adding water. The prepared medium was appropriated in aliquots of fifty (50) ml in twenty one (21) clean two hundred and fifty (250) ml conical flasks. Sets of three jars were balanced by including either hydrochloric acid (0.1M HCl) or sodium hydroxide (0.1M NaOH) (Saha *et al*., 2008) to reach the required pH of 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0. The pH was measured utilizing electrical pH meter (Jenway 3305) preceding sanitization in autoclave at 121°C

Inoculation and mycelial development of *A. parasiticus* in the broth media

A two (2) millimeter disc of *Aspergillus parasiticus* culture was placed in every flask. The flasks having culture were incubated at room temperature (27 ± 3°C) for 7 days. The cultured media were poured and independently sieved using weighted Whatman filter paper No.1 to recover mycelia. The mycelia were dried in oven at 800°C. The dried mycelia were measured (g) utilizing electrical mettle balance (Sibounnavoung *et al.,* 2009). The average value of the triplicates was noted.

Preparation of potato dextrose agar media of varying pH levels

Potato dextrose agar (PDA) was prepared with seven (7) various pH levels by adding 20g agar (solidifying agent) and prepared up to one liter with water. pH was balanced by adding either hydrochloric acid (0.1M HCl) or sodium hydroxide (0.1M NaOH) for desired pH (Saha *et al*., 2008). pH was measured by electrical pH meter (Jenway 3305) before sterilization in autoclave at 121°C. Disinfected media were poured in ten (10) cm diameter sterile Petri dishes in triplicate. Media was allowed to solidify and cool.

Inoculation and Sporulation of *A. parasiticus* in the agar media

Aspergillus parasiticus two (2) millimeter was obtained from the developing edges of cultured PDA colonies by using sterilized plug borer. Agar plugs were swapped to PDA plates (one for each plate) at different pH levels and incubated at temperature 27 ± 3°C for seven days (Carlos and Josep. 2012). Every treatment was in triplicate. Spores in each treated Petri dish was washed in 20 ml water by using camel hair brush into test tube. 0.2ml drop of this solution was set in the hemocytometer and counted under compound light microscope (Sibounnavoung *et al.,* 2009). Quantity of spores per ml was counted by given formula (Abubakar *et al.,* 2013)

Number of spores per ml =
Average spores counted × (25 × 10^4) × 20 (dilution factor).

Effect of light

Impact of light on *Aspergillus* spp. was observed as described by Afolayan *et al.,* (1997). Sterilized replicate plates containing Czapek-dox agar medium were inoculated aseptically and incubated at 28 ±2°C. Development was recorded under three different light conditions: complete darkness, nonstop light and alternative light/haziness (12hr: 12hr) in triplicate. Linear development of the fungal mycelium was measured every day from the range of inoculation along four (4) diameters and mean were noted.

Effect of relative humidity

Impact of RH on *Aspergillus* spp. was estimated at 32.5%, 50.5%, 85.0% and 100%. These conditions were planned by setting two hundred and fifty ml of saturated solutions of salts. *Aspergillus* spp. were inoculated on the Czapek-dox agar medium in triplicate and set in the desiccators. Average radial development of fungus was noted day by day until filled the Petri dishes.

Effect of temperature

Impact of temperature on *Aspergillus* spp. was observed in incubators at 30°C, 35°C and 40°C. Plates were inoculated with the related *Aspergillus* spp. independently as described by Shehu and Bello (2011) and then incubated at three different temperatures. Linear mycelial development was recorded daily and mean of three replicates was taken.

Statistical analysis

Data obtained from different pH levels for the growth of fungus were analyzed using one way Analysis of Variance (ANOVA) and the group means were compared by Duncan Multiple Range Test (DMRT) using the Statistical Package for Social Science (SPSS). Effect of light, humidity and temperature on the growth of fungus were analyzed using Pearson's x^2 distribution (chi-squared).

RESULTS AND DISCUSSION

Effect of various pH levels on dry mycelial weight of *Aspergillus parasiticus*

Results showed that *A. parasiticus* grows in acidic, neutral as well as in weak alkaline medium (pH 4.0 - 8.0) (Table 1). Highest mean of dried mycelial weight (365.67mg) was obtained at pH 4.0, followed by 353.3mg at pH 7.0 while lowest mycelia dry weight (302.73mg) was obtained at pH 10.0 broth medium ($P < 0.05$).

Effect of different pH levels on sporulation of *Aspergillus parasiticus*

pH 5.0 produced the highest spores per ml (8.243 × 10^7),

followed by pH 7.0 (7.677×10^7), while lowest development of spores' (2.893×10^7) was recorded at pH 10.0 (Table 2).

Effect of light

Growth under complete darkness was higher on first day of incubation in *A. candidus* and *A. niger* (Table 3), while *A. flavus* and *A. fumigatus* showed the highest growth under continuous light (Table 4). Significant growth ($P < 0.05$) was recorded under alternative light/darkness in all *Aspergillus* spp. by the end of 3rd day, until the end of day 6 (Table 5) ($P < 0.05$).

Table 1: Effect of various pH levels on dry mycelial mass of cultured *Aspergillus parasiticus.*

pH	(Mean ± Standard Deviation) mg
4.0	365.67±3.024[a]
5.0	362.97±2.511[a]
6.0	362.90±2.751[a]
7.0	363.30±27.214[a]
8.0	360.30±8.810[a]
9.0	340.77±21.795[a]
10.0	312.73±17.043[b]

*****Means in the same rows having different superscripts show significant differences from each other (P<0.05).

Table 2: Effect of various pH levels on per ml spore count of cultured *Aspergillus parasiticus.*

pH	(Mean× 10^7 ± Standard Deviation)
4.0	5.500±0.500[ac]
5.0	8.243±0.764[b]
6.0	4.893±0.577[c]
7.0	7.677±1.528[b]
8.0	4.343±0.764[ac]
9.0	3.363±0.288[ad]
10.0	2.893±0.288[d]

*****Means in the same rows having different superscripts show significant differences from each other (P<0.05).

Effect of relative humidity

Higher RH% significantly increased the growth of *Aspergillus* spp. 32.5% favored the growth of *A. niger* (Table 6), however, the growth in other *Aspergillus* spp. was significantly increased at 50.5%, 85.0% and 100% (Table 7, 8 and 9), shows that the *Aspergillus* species require high relative humidity for proper growth.

Effect of temperature

All *Aspergillus* species showed the best growth at 30°C and 35°C (Table 10, 11). At 40°C, only *A. fumigatus and A. flavus* showed significant growth (Table 12) (P<0.05).

Development and sporulation of *A. parasiticus* demonstrated that the fungal growth is tolerant of acidic and neutral conditions while, these attributes are altogether stifled by alkaline condition. These outcomes are inline with the results that growth of mycelia is influenced by pH of media (Abubakar *et al*., 2013, Sibounnavoung *et al.*, 2009). Filamentous fungi are not tolerant to acidic pH and mostly have an ideal pH around 5.0 and 6.0 for cell development and a few metabolic activities (Rosfarizan *et al*., 2000). Usually, a wide range of pH for development in *A. parasiticus* concerning dry mycelia weight and sporulation from 4.0 to 9.0 and 4.0 to 8.0 pH, respectively.

A. parasiticus has dry mycelia weight 14.89%.between 4.0 and 10.0 pH, as reported by (Kaiser *et al*., 2005) at pH 10.3 on *Alternaria solani* (42.8%), *Phytophthora capscici* (17.4%) and *P. cinnamomi* (12.6%) separately. At pH 11.7, the developments in these fungal spp. were completely restricted. Sporulation of *A. parasiticus* at different pH levels was similar as described by (Abubakar *et al*., 2013, Zhao *et al.*, 2010), who reported that pH 5.0 - 8.0 was good for conidial generation of *Diplocarpon mali,* similarly George *et al*., (1959) described that lower pH (4.5) don't influence spores generation stage, verified by Swe *et al*., (2009) that higher the alkalinity in media lower the colony counts / ml. This shows although certain alkaline medium (pH 8.0 and 9.0) may support the spore development of *A. parasiticus,* higher pH values from 10 have a tendency to reduce its sporulation.

Table 3: Comprehensive darkness effect on the linear growth of *Aspergillus* Species.

Aspergillus species	1st Day	2nd Day	3rd Day	4th Day	5th Day	6th Day	7th Day
A. candidus	0.02	1.62	1.05	0.48	0.06	0.67	1.18
A flavus	0.017	0.002	0.028	0.303	0.124	0.018	0.254
A fumigatus	0.096	0.298	0.622	0.984	0.231	0.463	2.925
A niger	0.047	0.547	0.058	0.159	0.015	0.133	0.539

Table 4: Uninterrupted Light Effect on the linear growth of *Aspergillus* Species.

Aspergillus species	1st Day	2nd Day	3rd Day	4th Day	5th Day	6th Day	7th Day
A. candidus	0.03	1.68	1.65	0.28	0.04	0.315	1.12
A flavus	0.033	0.038	0.122	0.117	0.136	0.001	0.202
A fumigatus	0.012	0.397	0.722	0.788	0.169	0.434	2.604
A niger	0.000	0.568	0.013	0.199	0.023	0.086	0.445

Table 5: Alternative light and darkness effect on the linear growth of *Aspergillus* Species.

Aspergillus species	1st Day	2nd Day	3rd Day	4th Day	5th Day	6th Day	7th Day
A. candidus	0.12	1.82	0.715	0.48	0.10	0.153	1.19
A flavus	0.001	0.027	0.009	0.708	0.136	0.063	0.152
A fumigatus	0.028	0.174	0.153	0.864	0.054	0.948	2.858
A niger	0.035	1.056	0.009	0.456	0.039	0.166	0.312

Table 6: Effect of relative humidity of 32.5% on the linear growth of *Aspergillus* Species.

Aspergillus species	1st Day	2nd Day	3rd Day	4th Day	5th Day	6th Day	7th Day
A. candidus	0.22	0.42	0.27	0.28	0.08	0.02	0.53
A flavus	0.003	0.149	0.187	0.104	0.003	0.443	0.016
A fumigatus	0.472.	0.032	0.007	0.017	0.019	0.022	0.262
A niger	0.087	0.008	0.273	0.207	0.008	0.032	0.518

Table 7: Effect of relative humidity of 50.5% on the linear growth of *Aspergillus* Species.

Aspergillus species	1st Day	2nd Day	3rd Day	4th Day	5th Day	6th Day	7th Day
A. candidus	0.05	0.37	1.02	0.28	0.003	0.29	1.52
A flavus	0.019	0.612	0.007	0.093	0.003	0.078	0.132
A fumigatus	0.087	0.652	0.577	0.005	0.022	0.489	0.267
A niger	0.019	0.902	0.298	0.002	0.003	0.456	0.087

Table 8: Effect of relative humidity of 85.5% on the linear growth of *Aspergillus* Species.

Aspergillus species	1st Day	2nd Day	3rd Day	4th Day	5th Day	6th Day	7th Day
A. candidus	0.02	0.68	1.78	0.44	0.02	0.84	1.73
A flavus	0.272	0.009	0.045	0.056	0.036	0.028	0.038
A fumigatus	0.362	1.862	1.454	0.288	0.045	0.502	2.782
A niger	0.002	0.058	1.245	0.052	0.012	0.226	0.663

Table 9: Effect of relative humidity of 100% on the linear growth of Aspergillus Species.

Aspergillus species	1st Day	2nd Day	3rd Day	4th Day	5th Day	6th Day	7th Day
A. candidus	2.62	0.39	0.65	0.65	0.03	1.69	1.38
A flavus	0.108	0.007	0.015	0.213	0.114	0.049	0.122
A fumigatus	1.025	3.018	1.648	0.083	0.038	1.262	2.068
A niger	0.022	0.537	0.389	0.094	0.043	0.598	0.797

Table 10: Effect of temperature (30°C) on the linear growth of *Aspergillus* Species.

Aspergillus species	1st Day	2nd Day	3rd Day	4th Day	5th Day	6th Day	7th Day
A. candidus	0.17	2.04	0.81	0.55	0.03	1.22	0.88
A flavus	0.009	0.001	0.038	0.588	0.094	0.094	0.213
A fumigatus	0.035	0.165	0.989	0.094	0.102	2.858	2.412
A niger	0.009	0.553	0.001	0.391	0.023	0.213	0.008

Table 11: Effect of temperature (35°C) on the linear growth of *Aspergillus* Species.

Aspergillus species	1st Day	2nd Day	3rd Day	4th Day	5th Day	6th Day	7th Day
A. candidus	0.12	2.62	0.89	0.69	0.000	0.88	2.67
A flavus	0.003	0.087	0.222	0.001	0.042	0.012	0.002
A fumigatus	0.022	0.162	0.013	0.034	0.000	0.015	0.002
A niger	0.001	0.156	0.033	0.116	0.039	0.043	0.002

Table 12: Effect of temperature (40°C) on the linear growth of *Aspergillus* Species.

Aspergillus species	1st Day	2nd Day	3rd Day	4th Day	5th Day	6th Day	7th Day
A. candidus	0.38	0.23	0.12	1.05	0.02	0.01	1.13
A flavus	0.133	0.195	0.643	0.269	0.002	0.049	0.491
A fumigatus	0.022	0.402	1.143	0.114	0.072	0.067	0.451
A niger	0.537	0.229	0.922	0.838	0.066	0.027	0.899

Growth under complete darkness was higher on day first in *A. candidus* and *A. niger,* while *A. flavus* and *A. fumigatus* showed highest growth under continuous light. Significant growth was noted under alternative light/darkness in all the *Aspergillus* species from 3rd day, till 6th day ($P<0.05$), agreed by Shehu and Bello (2011) that light seemed to have no beneficial impact on vegetation and conidiation of *Aspergillus* species.

Growth of the *Aspergillus* species enhanced by higher RH %, *i.e.* 50.5%, 85.0% and 100%, while lowest RH%, i.e. 32.5% only supported *A. niger,* as reported by Muhammad *et al*., (2004) who stated that low atmospheric RH% is frequently an element that reduces development of fungi. Growth of fungi under high RH % recommends to avoid conditions lower than 55% relative humidity agreed by Shehu and Bello (2011).

All *Aspergillus* species showed best growth at 30°C and 35°C temperature, only *A. fumigatus* and *A. flavus* survived at At 40°C, supported by Shehu and Bello (2011), Moss (2002) and Oladiran and Iwu (1993). 30°C temperature and 70-90% RH are ideal conditions for the development of *A. niger* and *A. flavus.* The positive fungal responce to high RH% and a temperature range of 30-35°C suggests to avoid storage of feedstuff under natural atmospheric conditions.

CONCLUSION

This study highlights optimal growth requirements for *Aspergillus* spp. a serious menace for the animal health and feed industry of Pakistan. Keeping a close check on these conditions in store houses and other feed manufacturing units will decrease *Aspergillus* contamination, ultimately raise animal health status. In this manner, certain alkaline medium can be investigated to restrain the mycelia development and sporulation of the fungi with a specific end goal to keep its harms away from our yield.

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