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Effect of Environmental Factors on the *Growth* of *Aspergillus Species* Associated with Stored Millet Grains *in Sokoto.*

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Abstract: Fungi constitute a major problem in the storage of agricultural products especially cereals. In the present study, the effects of light, relative humidity and temperature on the growth of *Aspergillus* species (*Aspergillus candidus, A. flavus, A. fumigatus, A. niger* and *A. oryzae*) associated with stored millet grains were investigated. Light stimulus had no significant effect on the mycelial growth of the fungi. Of the relative humidity regimes tested, 85% and 100% were the most favourable for the growth of *Aspergillus* species. Growth was generally poor at 32.5 and 50.5% relative humidities. There were significant ($p < 0.05$) differences in the growth of *Aspergillus* species with respect to temperature regimes. Generally, high growth was obtained under incubation temperatures of 30^{o}C and 35^{o}C. Cereals in general and millet grains in particular exposed to high humidity and temperature range of 30^{o}C and 35^{o}C may be susceptible to contamination by *Aspergillus* species.
Keywords: *Aspergillus* species*;* environmental conditions; millet; storage.

INTRODUCTION

Pearl millet [*Pennisetum glaucum* (L.) R. Br] is one of the most important cereal crops in Nigeria. It is grown mainly in the north, where it forms a staple food. The stalks are valuable building materials, fuel and livestock's feed. With an annual production figure of 7.7million metric tons of millet Nigeria ranked second only to India (FAO, 2008). Millet grains which are not consumed soon after harvest are often stored for many months to be sold or consumed later. While in storage, the grains may be susceptible to infection by a variety of fungi. Infection of grains by fungi results in reduced germination, visible mould discolouration, chemical and nutritional changes (Ehrlich, 2007). Fungi of the genus *Aspergillus* are common contaminants of food and feed stuffs, many species are saprobes and are found in a variety of habitats and are ubiquitous agents of decay. There are 132- 200 species of Aspergillus. Several Aspergillus species have been found associated with production of mycotoxins of public health importance (Ehrlich, 2007). As such, the occurrence of Aspergillus secondary metabolites in food stuffs such as millet grains is becoming an increasing environmental concern. The presence of *Aspergillus* species in stored millet may pose a threat to the health of both humans and livestock. In order to effectively reduce the infection of grains by fungi especially, toxigenic Aspergillus, it is important to identify them as rapidly as possible and the environmental factors which affect their growth and develovement be determined.

The present paper reports on the influence of environmental factors on the growth of *Aspergillus* species associated with millet grains in Sokoto, north-western Nigeria.

MATERIALS AND METHODS

Sample collection: Twenty samples of freshly harvested millet seeds were collected between August, 2009 and May, 2010 from five local government areas in Sokoto state north- western Nigeria. After harvest the grains were put in 60kg jute sacks commonly used for grain storage in the area. The sacks were kept for 10 months in a well-ventilated room. Small portion of the stored seeds (100g) were collected from various points of each sack for the isolation of associated fungi.

Isolation of fungi from millet grains

Freshly harvested and stored millet grains were surface sterilized by immersing in 1% sodium hypochlorite solution for 10 minutes. The grains were then rinsed with sterile distilled water and blotted dry using sterile No 1.Whatman filter paper. Ten seeds each of freshly harvested and stored millet were inoculated on freshly prepared potato dextrose agar (PDA) plates and then incubated at 28 $\pm 2^0$C for seven days. Emerging fungal colonies were continuously sub-cultured on potato dextrose agar plates to obtain pure cultures of the isolates. The fungi were identified based on cultural and morphological features using binocular microscope (Barnett and Hunter, 1998; Labbe and Garcia, 2001).

Effect of Light on Growth

The effect of light on the growth of *Aspergillus* species was determined using a modified technique of Afolayan *et al*. (1997). Sterilized replicate plates containing Czapek-dox agar medium were inoculated aseptically with actively growing associated *Aspergillus* species and incubated at 28 $\pm 2^0$C. The growth was studied under three light regimes: continuous darkness, continuous light and alternating light/darkness (12hr:12hr). Linear growth of the fungal mycelium was measured daily from the area of inoculation along four diameters and the mean of these values were recorded. Three replications were prepared for each treatment.

Effect of Relative Humidities (RH %) on Growth

The effect of relative humidity on the growth of *Aspergillus* species was determined in desiccators set at 32.5%, 50.5%, 85.0% and 100%. These conditions were designed by placing 250 ml of saturated solutions of salts in accordance with Winston and Bates (1960). The *Aspergillus* species were inoculated on the Czapek-dox agar medium in three replicate plates and placed in the desiccators. Average radial growth of the fungi were taken daily until when the Petri dishes were filled up.

Effect of Temperature on Growth

The effect of temperature on the growth *Aspergillus* species was determined in incubators set at 30^0C, 35^0C and 40^0C. The media plates were inoculated *with* the associated *Aspergillus* species separately as described in other conditions of growth. These were then incubated at the three temperature regimes. Linear mycelial growth was measured daily and mean of three replicate plates was taken as the growth rate for each fungal species.

Data obtained were analyzed using Pearson's X^2distribution (chi-squared) to assess significant differences at 0.05% level of probability (Gomez and Gomez 1984).

RESULTS AND DISCUSSION

Five *species of Aspergillus* were isolated from the stored grains. These are A. *candidus, A. flavus, A. fumigatus, A. niger and A. oryzae* with *A. flavus and A. niger as* the most frequently encountered species having 30% and 29.5% frequency of occurrence respectively (Table 1). The fungi may have colonized the grains during production in the field, transportation or storage. The variation in the frequency of their occurrence reflects differences in the inoculum density in the area or the prevailing environmental conditions favouring the growth of the fungi. The fungal organisms isolated from stored millet grains in the present study are known to be spoilage organisms associated with many agricultural products including cereals, fruits and nuts (Labbe and Garcia, 2001; Muhammad *et al*., 2004; Francisca *et al*., 2007). Growth under total darkness was higher on day 1 of incubation in *A. candidus, A. niger and A. oryzae* (Table 2), while *A. flavus* and *A. fumigatus* recorded the highest growth under continuous light (Table 3). Significant growth was recorded under alternative light/darkness regime in all the *Aspergillus* species at the end of day 3, which continues until the end of day 6 (Table 4). Generally, there were no significant differences in both regimes at 0.05% level of probability. This finding is in consonance to the observations made by Parry (1990) that light appeared to have no positive effect on the vegetative growth and conidiation of *Aspergillus* species.

Higher relative humidity significantly increased the growth of the *Aspergillus* species. The lowest relative humidity condition of 32.5% favoured only the growth of *A. niger* as shown in Table 5, however, the growth in other *Aspergillus* species was significantly increased by the relative humidity conditions of 50.5%, 85.0% and 100% (Tables 6,7,8). The growth shows a linear increase in all the treatments with time indicating that the *Aspergillus* species require high relative humidity for good growth. This is in agreement with the findings of Muhammad *et al.* (2004) that low ambient relative humidity is often a factor that limits growth of fungi on foods. The growth response of the fungi to high relative humidity suggests the need of storing millet grains under ambient environmental conditions lower than 55% relative humidity.

All *Aspergillus* species produced the best growth at the selected temperatures of 30^0C and 35^0C (Table 9, 10). At 40^oC, only *A. fumigatus and A. flavus* showed significant growth (Table 11). There were significant differences in all the treatments ($P>0.05$). Temperature was found to influence the growth of the isolated fungi. The optimum temperatures for growth of the *Aspergillus* species associated with millet are 30 and 35^oC. Only *A. fumigatus* appeared to have been influenced by incubation temperature of 40^0C. This observation is supported by the report of Moss (1989). Similarly, Oladiran and Iwu (1993) reported a temperature of 30^oC and a relative humidity range of 70-90% to be optimal conditions for the growth of A. *niger* and A. *flavus.* The growth response of the fungi to high relative humidity and a temperature range of 30-35^0C suggests the need of storing millet grains under ambient environmental conditions lower than 55% relative humidity and a temperature of 40^0C or above.

CONCLUSION

It can be concluded that *Aspergillus* species can grow and metabolise food under favourable relative humidity and temperature conditions causing spoilage or biodeterioration of the affected products, Therefore, in future efforts should be taken to store millet under dry conditions that are not favourable to *Aspergillus* growth, and to further find effective control measures to curtail the activities of these fungi as their association with stored grains may be responsible for grain loss and health hazards as a result of mycotoxin contamination.

Table 1: *Aspergillus* Species Isolated from Stored Millet Grains.

Local Gov't	*A. candidus*	*A. flavus*	*A. fumigatus*	*A. niger*	*A. orzae*	*Total spps*
Wammako	5	12	5	11	5	38
Tambuwal	3	12	12	12	4	43
Tureta	2	12	3	12	6	35
Illela	4	12	6	12	4	30
Isa	0	12	12	12	5	41
Total	14	60	40	12	24	197
Occurrence (%)	7.0	30.0	20.5	29.5	12.0	100

Table 2: Effect of Total Darkness Regime on the Linear Growth of *Aspergillus* Species Isolated from Stored Millet Grains.

	$(O-E)^2/E$							$\sum(O-E)^2/E$
	DAYS							
A. species	**1**	**2**	**3**	**4**	**5**	**6**	**7**	17.62
A. candidus	0.01	1.61	1.04	0.47	0.05	0.66	1.17	
A flavus	0.016	0.001	0.027	0.302	0.123	0.017	0.252	
A fumigatus	0.094	0.297	0.620	0.983	0.230	0.460	2.922	
A niger	0.045	0.545	0.056	0.158	0.014	0.131	0.538	
A oryzae	0.084	0.641	2.114	0.245	0.60	0.538	1.207	

O = observed variable, E = expected variable

Table 3: Effect of Continuous Light regime on the linear Growth of *Aspergillus* Species Isolated from Stored Millet Grains.

	$\left(O-E\right)^2/E$							$\sum\left(O-E\right)^2/E$
				DAYS				
A. species	**1**	**2**	**3**	**4**	**5**	**6**	**7**	15.26
A. candidus	0.02	1.69	1.63	0.29	0.03	0.314	1.10	
A flavus	0.032	0.039	0.120	0.115	0.135	0.000	0.200	
A fumigatus	0.011	0.396	0.720	0.786	0.168	0.432	2.603	
A niger	0.000	0.569	0.011	0.198	0.022	0.085	0.444	
A oryzae	0.001	0.576	2.734	0.200	0.085	0.519	0.028	

Table 4: Effect of Light/Darkness Regime on the Linear Growth of *Aspergillus* Species Isolated from Stored Millet Grains.

	$\left(O-E\right)^2/E$							$\sum\left(O-E\right)^2/E$
				DAYS				
A. species	**1**	**2**	**3**	**4**	**5**	**6**	**7**	30.11
A. candidus	0.10	1.80	0.714	0.47	0.09	0.150	1.17	
A flavus	0.000	0.026	0.008	0.707	0.135	0.061	0.150	
A fumigatus	0.027	0.172	0.150	0.862	0.053	0.947	2.857	
A niger	0.034	1.054	0.007	0.454	0.038	0.164	0.310	
A oryzae	0.092	10.10	2.603	0.010	0.326	0.326	1.800	

Table 5: Effect of Relative Humidity of 32.5% on the Linear Growth of *Aspergillus* Species Isolated from Stored Millet Grains

	$\left(O-E\right)^2/E$							$\sum\left(O-E\right)^2/E$
				DAYS				
A. species	**1**	**2**	**3**	**4**	**5**	**6**	**7**	4.519
A. candidus	0.20	0.40	0.26	0.26	0.07	0.01	0.51	
A flavus	0.002	0.148	0.186	0.103	0.002	0.441	0.014	
A fumigatus	0.470.	0.030	0.005	0.016	0.018	0.021	0.261	
A niger	0.086	0.007	0.272	0.206	0.009	0.031	0.519	
A oryzae	0.002	0.030	0.020	0.058	0.093	0.038	0.121	

Table 6: Effect of Relative Humidity of 50.5% on the Linear Growth of *Aspergillus* Species Isolated from Stored Millet Grains.

	$\left(O-E\right)^2/E$							$\sum\left(O-E\right)^2/E$
				DAYS				
A. species	**1**	**2**	**3**	**4**	**5**	**6**	**7**	10.403
A. candidus	0.04	0.36	1.01	0.27	0.002	0.28	1.51	
A flavus	0.018	0.610	0.005	0.091	0.002	0076	0.131	
A fumigatus	0.085	0.650	0.576	0.004	0.020	0.487	0.266	
A niger	0.018	0.900	0.299	0.001	0.002	0.454	0.085	
A oryzae	0.220	0.360	0.955	0.033	0.006	0.238	0.342	

Table 7: Effect of Relative Humidity of 85.5% on the Linear Growth of *Aspergillus* Species Isolated from Stored Millet Grains.

	$(O-E)^2/E$							$\sum(O-E)^2/E$
	DAYS							
A. species	**1**	**2**	**3**	**4**	**5**	**6**	**7**	20.385
A. candidus	0.01	0.69	1.77	0.43	0.01	0.83	1.72	
A flavus	0.270	0.008	0.044	0.055	0.035	0.027	0.036	
A fumigatus	0.360	1.860	1.453	0.289	0.044	0.500	2.780	
A niger	0.001	0.059	1.244	0.050	0.011	0.225	0.662	
A oryzae	0.689	1.763	1.205	0.261	0.102	0.848	0.046	

Table 8: Effect of Relative Humidity of 100% on the Linear Growth of Aspergillus Species Isolated from Stored Millet Grains

	$(O-E)^2/E$							$\sum(O-E)^2/E$
	DAYS							
A. species	**1**	**2**	**3**	**4**	**5**	**6**	**7**	25.992
A. candidus	2.61	0.38	0.64	0.64	0.02	1.68	1.37	
A flavus	0.109	0.006	0.014	0.212	0.113	0.048	0.121	
A fumigatus	1.024	3.017	1.649	0.083	0.039	1.260	2.067	
A niger	0.020	0.536	0.388	0.093	0.042	0.599	0.796	
A oryzae	1.55	1.072	1.249	0.022	0.131	0.868	0.098	

Table 9: Effect of Temperature Condition of 30°C on the Linear Growth of *Aspergillus* Species Isolated from Stored Millet Grains.

	$(O-E)^2/E$							$\sum(O-E)^2/E$
	DAYS							
A. species	**1**	**2**	**3**	**4**	**5**	**6**	**7**	21.896
A. candidus	0.16	2.03	0.80	0.54	0.02	1.20	0.89	
A flavus	0.008	0.000	0.039	0.589	0.093	0.093	0.212	
A fumigatus	0.034	0.164	0.988	0.093	0.101	2.857	2.410	
A niger	0.008	0.552	0.000	0.390	0.022	0.212	0.007	
A oryzae	0.384	0.716	3.337	0.033	0.085	0.800	1.102	

Table 10: Effect of Temperature Condition of 35°C on the Linear Growth of *Aspergillus* Species Isolated from Stored Millet Grains.

	$(O-E)^2/E$							$\sum(O-E)^2/E$
	DAYS							
A. species	**1**	**2**	**3**	**4**	**5**	**6**	**7**	31.525
A. candidus	0.10	2.60	0.88	0.68	0.000	0.87	2.66	
A flavus	0.002	0.086	0.220	0.000	0.040	0.011	0.001	
A fumigatus	0.021	0.160	0.011	0.033	0.000	0.014	0.001	
A niger	0.000	0.155	0.031	0.115	0.038	0.042	0.001	
A oryzae	0.242	0.076	2.411	0.295	0.142	0.600	0.868	

Table 11: Effect of Temperature of 40^oC on the Linear Growth of *Aspergillus* Species Isolated from Stored Millet Grains**.**

	$(O-E)^2/E$							$\sum(O-E)^2/E$
				DAYS				
A. species	**1**	**2**	**3**	**4**	**5**	**6**	**7**	13.13
A. candidus	0.37	0.22	0.10	1.04	0.01	0.01	1.13	
A flavus	0.132	0.194	0.642	0.268	0.001	0.048	0.490	
A fumigatus	0.020	0.400	1.142	0.113	0.073	0.066	0.450	
A niger	0.535	0.228	0.920	0.839	0.065	0.026	0.898	
A oryzae	0.066	0.457	0.980	0.076	0.042	0.149	0.102	

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