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Effect of sodium chloride on the growth and aflatoxins` production of *Aspergillus flavus* Link isolated from poultry feedstuff samples

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Abstract: Seven genera and 14 species were collected from 20 poultry feed samples collected from Sohag City, Egypt. *Aspergillus flavus* was the most dominant species representing 53% of the gross total count of fungi. Twenty randomly-selected isolates of *A. flavus*, one from each sample, in addition to a reference strain (IMI 102135) were grown on 5 sodium chloride concentrations at 25 ±2°C to test their effect on some growth parameters of the isolates including macroscopic and microscopic features, in addition to aflatoxins production. The toxicity of cleaned crude extracts of the twenty fungal isolates and the reference strain against the green alga *Chlorella vulgaris* Bejerinck was assessed. Five isolates, in addition to the reference strain, were highly toxic, three moderately toxic and the rest were low. TLC revealed that in two high toxic isolates and the reference strain, aflatoxins B_1, B_2, G_1, and G_2 were detected, whereas aflatoxins B_1 and G_1 only were detected in the cleaned crude extract of nine other moderately and low toxic isolates. None of the isolates that had no biological toxicity on *Chlorella vulgaris* produced aflatoixns. High concentration of sodium chloride (10%) inhibited completely the growth and accordingly aflatoxins production of all fungal strains tested. Nonetheless; while the fungal growth was significantly enhanced on 2.5 and 5% concentrations, the production of aflatoxins was only inhibited on 5% of salt. The conidial heads of the reference strain of *A. flavus* turned to be occasionally columnar on 2.5% salt to mostly columnar uniseriate on 5% salt rather than being typically radiate, uni- and biseriate on 0% salt. The conidiophores were markedly increased in length on 5% salt (2 mm) by merely one fold, whereas the conidial heads were moderately increased in length three times (1.1 mm) more than those grown on 0% salt (346 µm). No considerable changes were noticed on the vesicle nor the conidial shape (globose to subglobose) or diameter (3.75 µm) on the different salt concentrations used.

Key words: *Aspergillus flavus* , aflatoxins, fungal growth, sodium chloride, feedstuff, *Chlorella vulgaris*

Introduction

Aspergillus is a large genus composed of more than 180 accepted anamorphic species with teleomorphs described in nine different genera (Pitt *et al.* 2000). This genus is subdivided into 7 subgenera, which are further divided into sections (Klich 2002). According to Raper and Fennell (1965) the macroscopic features of colonies *Aspergillus flavus* on Czapek's agar medium range between 3-7 cm per week, yellowish-green, provided with dense conidiophores; with uncolored to pinkish drab reverse. The conidial head is typically radiate, uni- and biseriate, most commonly 300 to 400 µm, smaller heads occasionally columnar up to 300 µm. Conidiophore heavy walled, uncolored, coarsely roughened, usually less than one mm in length but occasionally up to 2.5 mm. Vesicle subglobose or globose most commonly 25-45 µm in diameter. Conidiogenous cell uni- or biseriate. Conidia typically globose to subglobose, conspicuously echinulate, variable from 3.5 to 4.5 µm. Sclerotia when present, globose to subglobose and gradually changing from white to near black, 400 to 700 µm.

Aspergillus flavus is a saprobic fungus with a cosmopolitan distribution. It colonizes cereal grains and legumes. Pre-harvest infection can occur in the field with no symptoms while post-harvest rot is developed either during harvest or storage. In addition, many strains produce significant quantities of toxic compounds known as aflatoxins which when consumed are toxic to mammals (Agrios 2005). Aflatoxins, are secondary metabolites produced not only by *A. flavus,* but also by *A. parasiticus, A. nomius, A. tamarii*, *A. bombycis* and *A. pseudotamarii* (Kurtzman *et al.* 1987). Aflatoxins are acutely toxic, carcinogenic, teratogenic and mutagenic (Goldblatt 1969, Barnes 1970, Keyl and Booth 1971, Ciegler 1975). Numerous strategies have been proposed to eliminate, detoxify or reduce aflatoxins in foods and feed stuff (Thanaboripat 2002). Chemical inhibitors are used as the most effective way to control the problems caused by aflatoxins by preventing fungal growth in the susceptible substrate (Moreno-Martinez. *et al.* 2000) Ammoniation and sodium bisulfite have been used as chemical approaches to the detoxification of aflatoxins (Anderson 1983). The use of natural and cheep salts such as of sodium chloride and ammonium carbonate, prevented the growth of *A. flavus* and aflatoxin production in corn (Thanaboripat *et al*. 1992, Chitaree *et al.* 1993). The aim of this investigation was to study the mycobiota and their predominant species contaminating 20 feedstuff samples, the aflatoxins produced by 20 isolates of *A. flavus*, the most dominant species, and how much the salinity by sodium chloride would inhibit the growth parameters and inhibit the production of aflatoxins by these isolates comparable with the reference strain IMI 102135.

Materials and Methods

Determination of mycobiota of poultry feedstuff samples

Dilution plate method was used for isolating different fungal genera and species that contaminate 20

poultry feedstuff samples (500 gm each) collected from different commercial poultry stores in Sohag City. Czapek`s glucose agar medium was used as an isolation medium. Five replicate plates were incubated at 25±2°C for 5-7 days. The developed fungi were counted per gm sample**,** isolated and identified using the identification keys of Raper and Fennell (1965), Ainsworth (1971), Moubasher (1993) and Domsch *et al*. (2007).

Aflatoxins screening of *A. flavus* isolates

Twenty isolates of *A. flavus* of those emerged from the poultry feedstuff samples, in addition to a reference strain of *A. flavus* (IMI 102135), were used throughout this investigation. The isolates were cultivated on Czapek's glucose agar plates and incubated at 25 ±2°C for 10 days. At the end of incubation period, an agar plug technique (Sleigh and Timburg 1981) was employed for the extraction of aflatoxins that might be produced. The agar medium along with the fungal mycelia were cut into small pieces (1cm) and` transferred into a 250 ml Erlenmeyer flask containing 50 ml 96% methanol. The contents were shaken on a rotary shaker (200 r.p.m., 24 h) and filtered through Whattman filter paper No. 1 (Aboul-Nasr and Obied-Allah 2013, Aboul-Nasr *et al.* 2013). The extracted materials were washed using 25 ml of the same solvent, the extracts were combined, dried over anhydrous sodium sulphate, and left to evaporate under ambient temperature. Residues were transferred into vials and further evaporated to near dryness.

Crude extracts cleaning up procedure

Fungal crude extracts were immersed in one ml chloroform and transferred onto 14X0.8 cm column containing 25 gm Kiesel Gel 60,70/230 Silica Gel (MERK. Germany) and washed by 8 ml of n-hexane. Aflatoxins were eluted using 8 ml of 3:97 methanol-chloroform (AOAC 1984).

Biological assay of the toxicity of crude extracts of *A. flavus* isolates using *Chlorella vulgaris*

Three replicates of 10 µl of each clean crude extracts were transferred into test tubes containing 6 ml of *Chlorella vulgaris* culture under aseptic conditions, and the growth of algal cells was measured by their optical density (O.D. at 750 nm) after 48 hours using thermo scientific, evolution 160 UV-Vis, double beam spectrophotometer (Aboul-Nasr *et al*. 2013).

Thin layer chromatographic analysis (TLC)

For aflatoxins detection, the fungal crude extracts were dissolved in the minimal amount of 96% methanol. Ten µl of each crude extract along with aflatoxin standards (B_1 and G_1) were spotted on pre-coated silica gel plates (Sigma) and developed with toluene: chloroform: acetone (15:75:10 v/v). The plates were viewed under long wave UV lamp (365 nm). The retardation factor (R.f) for aflatoxins B_1 and G_1 on silica gel plate is around 0.55 and 43 respectively (El- kady and Moubasher 1982).

Effect of different concentrations of sodium chloride on the growth and aflatoxins production of *A. flavus*

a- Fungal growth and aflatoxins prodution

The 20 chosen isolates of *A. flavus*, in addition to the reference strain of *A. flavus* IMI 102135 were cultivated in 9 cm Petri dishes on Czapek's glucose agar medium supplemented with different concentrations of sodium chloride (0, 2.5, 5, 7.5 and 10 gm/l), three replicates for each, and incubated at 25±2°C for 7 days. The diameter in cm of the fungal growth was measured and aflatoxins analysis was accomplished according to Aboul-Nasr *et al*. (2013).

b- Fungal dry weight

Spore suspensions of the 20 chosen isolates of *A. flavus*, in addition to the reference strain IMI 102135 were prepared (10^7 spore/ml) using Neubraur haemacytometer. One ml of each spore suspension was transferred to three replicates of 250 ml Erlenmeyer flasks containing 100 ml of sterile Czapek's glucose broth supplied with 0, 2.5, 5, 7.5 and 10 gm/l of sodium chloride and incubated at 25±2°C for 7 days. The fungal mat was collected on pre-weighed Whattman No.1 filter papers and the dry weight of the fungal mat was determined.

c- Microscopic features of *A. flavus* IMI 102135

The strain was cultivated on Czapek's glucose agar medium supplemented with different concentrations of sodium chloride (0, 2.5,and 5) gm/l ,three replicates for each concentration, and incubated at 25±2°C for 5 days. The macroscopic and microscopic features including conidial head, conidiophore, vesicle, conidiogenous cell, and conidial diameter were determined.

Statistical Procedures

All data were analyzed according to the procedure of analysis of variance (ANOVA) corresponding to factorial experiment in randomized complete – block design. The means of the treatments were compared using the Least Significant Difference (LSD) at P = 1% level (Gomez and Gomez 1984).

Results and Disscusion

The mycological analysis of 20 poultry feedstuff samples collected from Sohag City on Czapek's agar medium at 25±2°C are summarized in Table (1). A total of 3902 colony forming units (cfu) per gm were obtained from all tested samples. Seven genera comprising 14 species were isolated and identified. *Aspergillus* (8 species) dominated the fungal genera isolated and occupied 90% of the total counts. *A. flavus* overwhelmed the recovered species representing 53% of the total count. *A. versicolor* came next followed by *A. terreus, A. niger, A. fumigatus* and *A. candidus* representing 17, 6, 6, 4 and 3 % of the total count respectively. The frequency of *Aspergillus* species ranged between 10-100% from which *A. flavus* and *A. niger* were isolated from 100% of the samples, followed by *A. terreus, A. candidus, A. fumigatus, A. versicolor, A. ustus* and *A. sydowii*.

Table (1): Total colony forming units per gm (CFU), CFU %, number of cases of isolation (NCI) and percentage frequency (F %) of fungi recovered from poultry feedstuff samples on Czapek`s agar medium, at 25±2°C.

Fungal species	CFU	CFU %	NCI	F %
Aspergillus candidus Link	112	2.87	14	70
A. flavus Link	2086	53.46	20	100
A. fumigatus Fresenius	160	4.10	10	50
A. niger van Tieghem	224	5.74	20	100
A. sydowii (Bainier and Sartory) Thom and Church	4	0.10	2	10
A. terreus Thom	256	6.56	16	80
A. ustus (Bain.) Thom & Church	24	0.62	6	30
A. versicolor (Vuillemin) Tiraboschi	680	17.43	10	50
Circinella muscae (Sorokin) Berlese and De Toni	150	3.84	12	60
Fusarium solani (Mart.) Sacc.	62	1.59	6	30
Mucor circinelloides Fresenius	32	0.82	6	30
Penicillium chrysogenum Thom	72	1.85	10	50
Rhizopus stolonifer (Ehrenb.) Vuillemin	32	0,82	4	20
Syncephalastrum racemosum Cohn ex Schroete	8	0.20	2	10
Total colony-forming units (CFUs)	3902	100		
Number of genera	7			
Number of species	14			

The other fungal species represented 10 % of the total fungal counts. Almost similar results were recorded by Abdel-Hafez *et al.* (1992), Costa and Scussel (2002), González Pereyra *et al.* (2008) and Youssef *et al.* (2008).

Biological assay of *A. flavus* crude extracts

Twenty fungal isolates (one per each sample), in addition to the reference strain IMI 102153 of *A. flavus* were chosen and their crude extract effects on optical density of *Chlorella vulgaris* cultures at 750 nm after 48 hours of incubation were compared with the control (Table 2). The bioassay showed high toxicity of the crude extracts of 5 isolates, in addition to the reference strain, whereas four and six isolates showed moderate and low toxicity respectively. Five fungal crude extracts showed no toxic effect on *C. vulgaris*. The use of optical density readings of the growth of *C. vulgaris* reflected a rapid, easy and effective tool to detect mycotoxins (Bean *et al.* 1992, Aboul-Nasr and Obied-Allah 2013 and Aboul-Nasr *et al.* 2013).

Aflatoxin analysis of *A. flavus* isolates using thin layer chromatography

Thin layer chromatographic (TLC) analysis of the fungal crude extracts revealed that two isolates (No. 14 & 20) of the highly toxic, in addition to the reference strain, could produce aflatoxins B_1, B_2, G_1, and G_2, whereas the moderately toxic isolates and 2 of the low toxic ones could produce aflatoxins B_1 and G_1 (Table 2). No aflatoxins were detected in the non-toxic isolates. Aflatoxin B1 is identified as the most potent carcinogenic substance naturally produced mainly by *A. flavus* and *A. parasiticus* (Squire 1981) and is classified by the International Agency of Research on Cancer as a group 1 human carcinogen (IARC 1982).

Table (2): Biological assay of toxicity using *C. vulgaris* cells and TLC analysis of the crude extracts of twenty isolates, and of the reference strain of *A. flavus*.

A. flavus isolates	OD*	Aflatoxins detected
IMI 102135	H	B_1,B_2,G_1 & G_2
1	H	B_1 & G_1
2	L	-
3	L	-
4	N	-
5	N	-
6	N	-
7	H	B_1 & G_1
8	M	B_1 & G_1
9	L	-
10	L	-
11	N	-
12	M	B_1 & G_1
13	L	B_1 & G_1
14	H	B_1, B_2, G_1 & G_2
15	H	B_1 & G_1
16	M	B_1 & G_1
17	L	B_1 & G_1
18	N	-
19	M	B_1 & G_1
20	H	B_1, B_2, G_1 & G_2

OD*: Optical density of C. *vulgaris* culture at 750 nm after 48 hrs; control and no toxicity (N) = 0.123 or more, low toxicity (L) = 0.131 -0.099, moderate toxicity (M) = 0.098-0.026 & high toxicity (H) = 0.025 or less.

Effect of sodium chloride concentration on growth parameters of *A. flavus* isolates

In this study, four concentrations of sodium chloride (2.5, 5, 7.5 and 10%), in addition to the control of Czapek's agar and broth media at 25±2 ºC for 7 days were used to investigate their ability to control the growth and aflatoxin production of the twenty isolates plus the reference strain of *A. flavus* IMI 102153. The results in Table (3) and Figures (1 & 2) showed that higher concentrations of sodium chloride (7.5 and 10%) inhibited dramatically the growth as well as the aflatoxins production of all fungal strains tested. On the other hand, it was obvious that the dry weight was significantly enhanced in case of 2.5 and 5% NaCl concentrations, while the aflatoxins production was inhibited in case of 5% salt and onwards. Numerous strategies have been proposed to eliminate, detoxify or reduce aflatoxins in food- and feedstuff (Thanaboripat 2002). One of the most effective ways to control the problems caused by aflatoxins is to prevent fungal growth in the substrate such as the use of chemical inhibitors to suppress the spore germination of the fungi, as well as the development of the fungus mycelium, in the substrate susceptible to contamination by these toxins (Moreno-Martinez *et al*. 2000). Previous studies showed that the use of sodium chloride and ammonium carbonate completely prevented the growth of *A. flavus* and aflatoxin production in corn (Thanaboripat *et al*. 1992, Chitaree *et al*. 1993). El-Gazzar *et al.* (1986) found that increasing the concentration of sodium chloride reduced the accumulation of aflatoxin and also induced a lag in growth. *A. flavus* and *A. parasiticus*. On the other hand, Malbrouk and El-Shayeb (1980) found that sodium chloride concentrations of 5-50 mg/l enhanced aflatoxin formation in a liquid medium.

Further investigation on the effect of sodium chloride concentrations on the macro and micro morphological features of the reference strain of *A. flavus* IMI 102153 grown on Czapek's agar medium provided with 0, 2.5 and 5% sodium chloride for 5 days at 25±2ºC revealed that the conidial heads which are typically radiate, uni-and biseriate on 0% salt turned to be occasionally columnar to mostly columnar in case of 2.5 and 5% salt respectively as shown in Figure (3). The conidiophores were noticeably increased in length on 5% salt (2 mm) by merely one-fold, whereas the conidial heads were drastically increased in length three times (1.1 mm) that of the control (346 µm). No considerable changes were noticed in the vesicle (globose to subglobose, 45 µm in diameter) or the conidial shape (globose to subglobose) or diameter (3.5 µm) at the different salt concentrations used.

Table (3): Effect of sodium chloride at various concentrations on the growth and aflatoxins production of twenty isolates in addition to the reference strain of *A. flavus* IMI 102135 on Czapek's agar and broth media at 25±2 ºC for 7 days.

A.flavus isolates No.	Diameter of colony / cm				Dry weigt / g				Aflatoxins detected	
	0%	2.5%	5%	7.5%	0%	2.5%	5%	7.5%	0%	2.5%
IMI 102135	4.2	5.1	5.5	1.5	0.225	0.360	0.577	0.031	+	+
1	4.0	4.5	4.5	1.5	0.219	0.359	0.603	0.014	+	+
2	4.5	5.0	5.0	1.5	0.242	0.315	0.590	0.013	-	-
3	4.2	4.7	5.5	2.0	0.243	0.367	0.613	0.021	-	-
4	5.0	4.6	6.0	1.8	0.259	0.347	0.617	0.014	-	-
5	4.1	4.3	5.5	1.7	0.251	0.369	0.687	0.033	-	-
6	4.0	5.0	5.9	2.0	0.257	0.360	0.598	0.054	-	-
7	4.0	4.9	5.6	2.1	0.260	0.318	0.602	0.072	+	+
8	4.0	4.7	5.7	2.2	0.263	0.378	0.613	0.061	+	+
9	4.3	4.6	5.0	2.0	0.287	0.397	0.577	0.059	-	-
10	4.0	4.5	6.1	1.8	0.245	0.366	0.659	0.067	-	-
11	4.0	4.7	5.5	1.5	0.248	0.359	0.629	0.075	-	-
12	3.9	4.8	5.5	1.7	0.267	0.389	0.619	0.071	+	+
13	4.3	4.3	5	1.6	0.219	0.317	0.593	0.051	+	+
14	4.2	4.5	5.6	1.4	0.245	0.363	0.589	0.035	+	+
15	4.1	4.8	5.8	2.0	0226	0.390	0.601	0.029	+	+
16	4.0	4.9	5.8	1.5	0.272	0.377	0.587	0.036	+	+
17	4.0	4.7	5.9	1.5	0.223	0.329	0.516	0.049	+	+
18	4.2	5.1	6.0	1.3	0.260	0.401	0.593	0.020	-	-
19	4.0	4.5	5.5	1.2	0.217	0.397	0.621	0.036	+	+
20	4.5	4.5	5.9	1.3	0.229	0.289	0.601	0.052	+	+

- No growth was detected for any of the 20 *A. flavus* isolates or the reference strain on 10%, while no aflatoxins were detected on 5%,7.5% and10% NaCl concentrations.

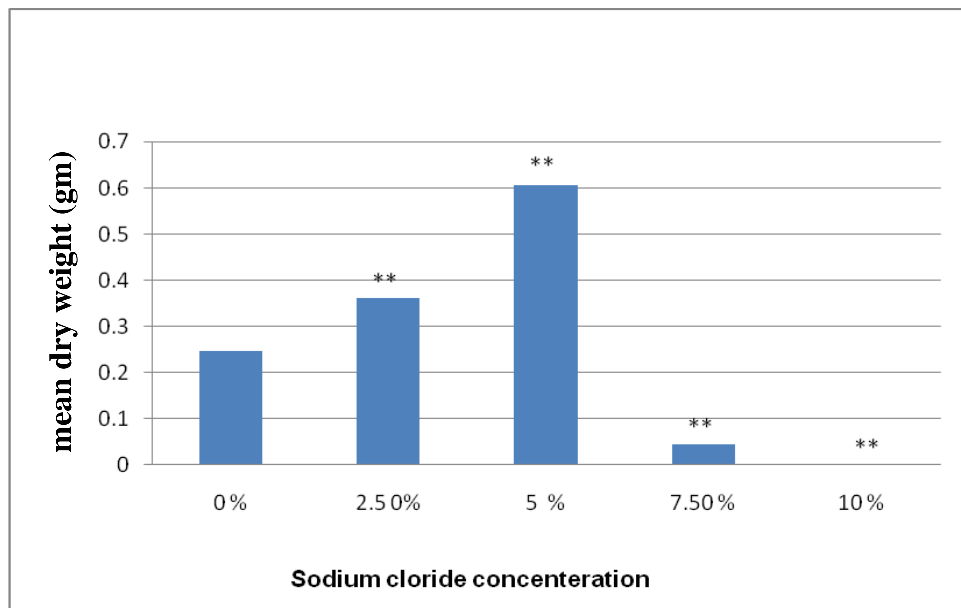

Figure (1): Effect of different concentrations of sodium chloride on the mean dry weight of all fungal isolates. **LSD = 0.0313 at P = 1% level .

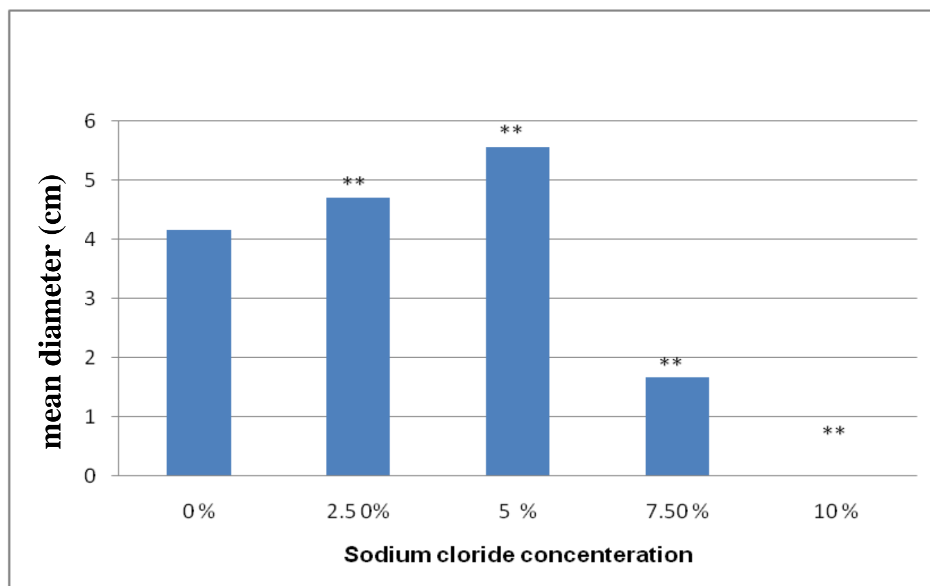

Figure (2): Effect of different concentrations of sodium chloride on the mean diameter of all fungal isolates. ** LSD = 0.4172 at P = 1% level

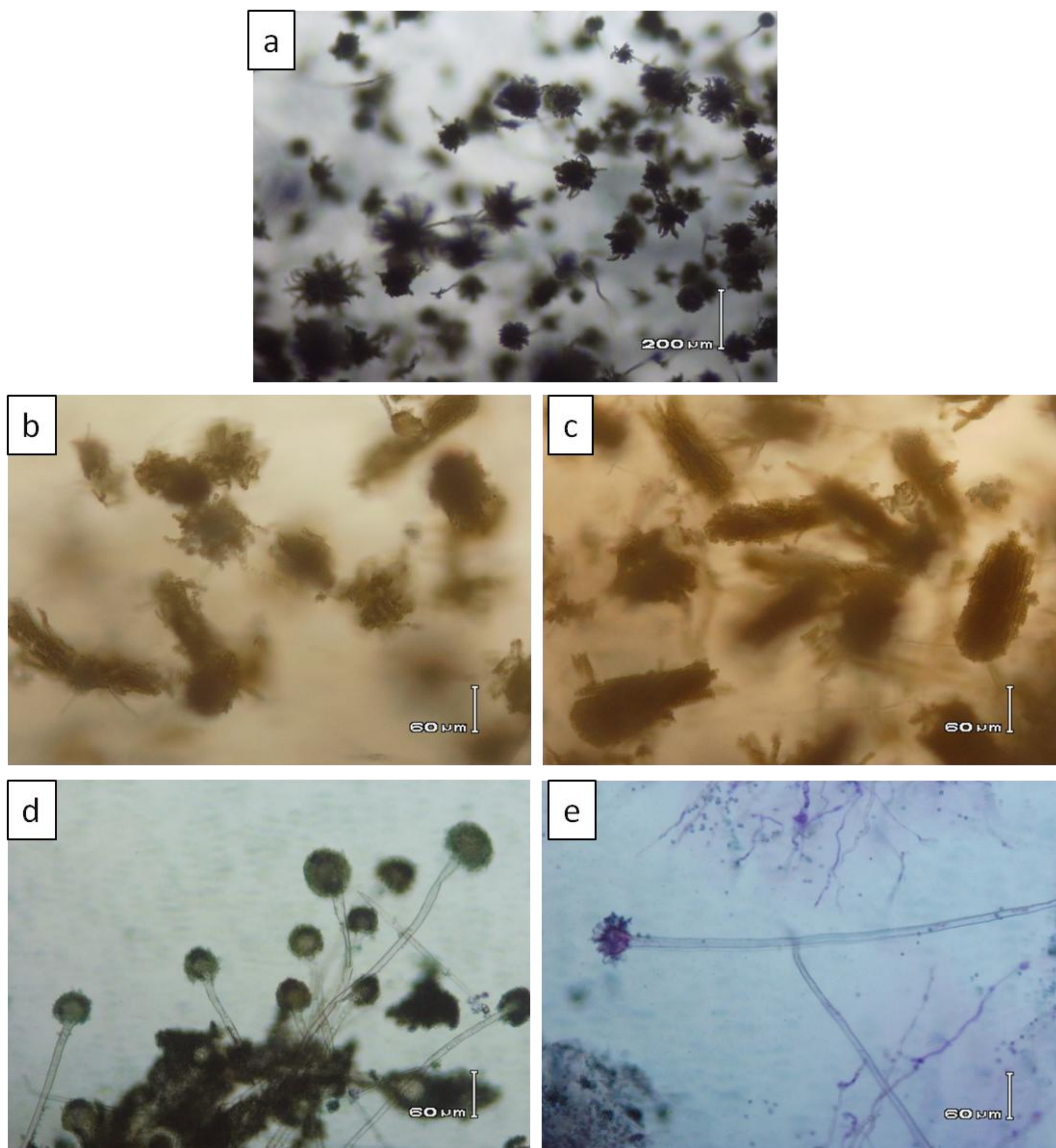

Figure (3): Conidial heads (a, b & c) of *A. flavus* IMI 102135 strain cultivated on Czapek's agar medium supplied with different concentrations of sodium chloride 0, 2.5,and 5 gm/l, and conodiophores (d & e) on 0 and 5% salt respectively.

Conclusion: The present investigation contributes an interesting result, that 2.5 and 5% sodium chloride concenteration induced noticeable conidial head and conidiophore morphogenesis of *A. flavus* IMI 102135. Also, both concentrations significantly enhanced the mean mycelial growth of the 20 *A. flavus* isolates and the reference strain, but 5% concentration completely inhibited aflatoxins production, wheareas 7.5 and 10% eliminated growth an accordingly aflatoxins production, a significant observations that might be beneficial for the purpose of elimination of the hazardous aflatoxins from food- and feedstuffs.

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